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ERRATA

Page 121, line 10 should read "description of the insect, of which all of the
early stages are here described and"
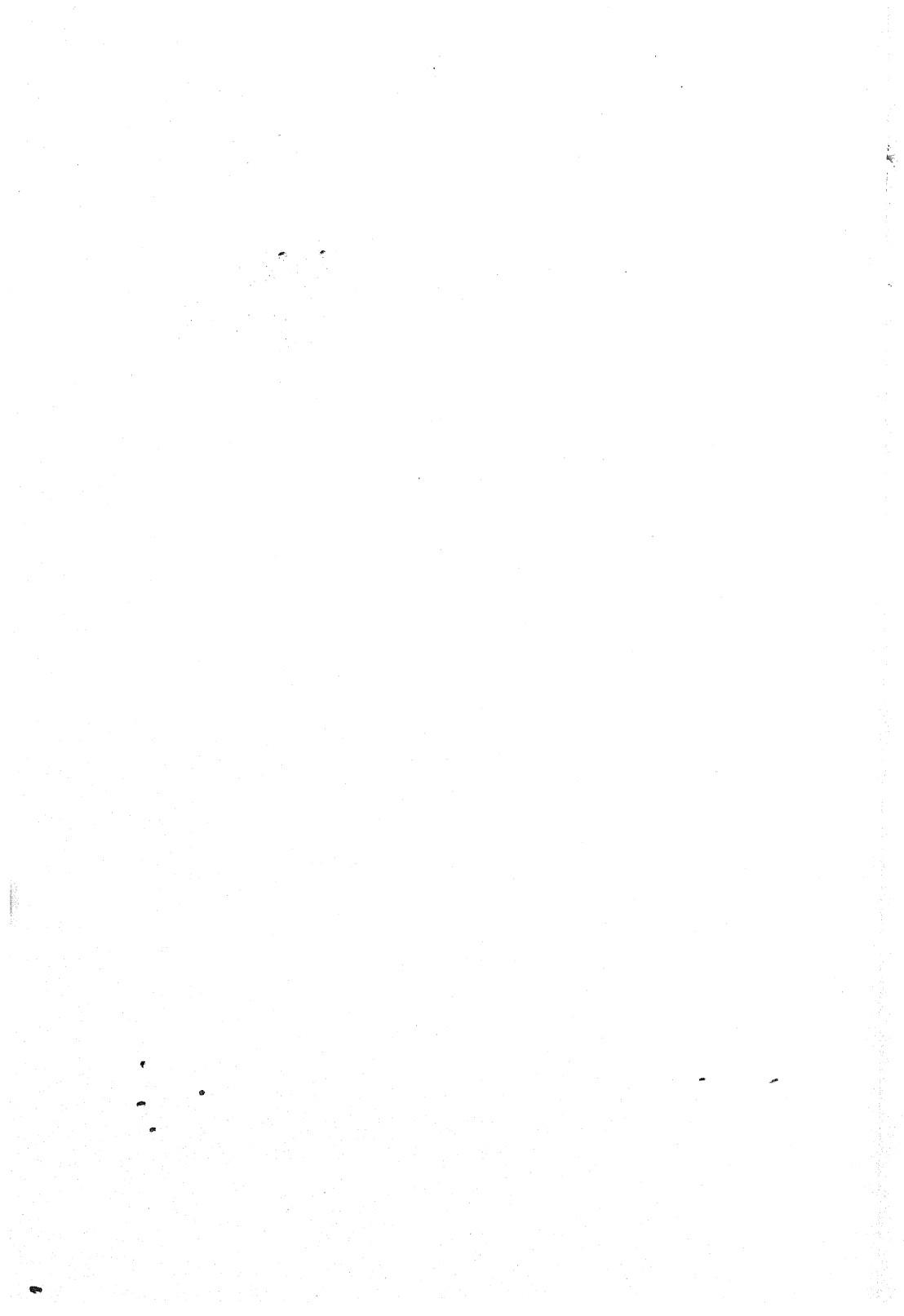
PECTIC ACID FROM THE COTTON FIBER[1]

STANTON A. HARRIS AND H. JEANNE THOMPSON

INTRODUCTION

The presence of pectin in the cotton fiber was reported by Schunck (10) in 1871. He stated that an alkaline extract of cotton, when acidified, gave a gelatinous precipitate which was chiefly pectic in nature, as shown by its characteristic appearance and properties. Leech (9), in an English patent applied for in 1916, gave a method for the extraction of pectin from vegetable fibers by the use of hydrochloric acid. Ehrlich and Haensel (5), in 1935, reported the extraction of pectin from ramie. Early in 1936, Anderson (2) reported the extraction of pectin from wood.

The present investigation was undertaken as part of the program for the study of the cementing material in the cotton fiber, as described by Farr and Eckerson (8). The present paper describes first the extraction of pectic acid from the mature cotton fiber, and second, its definite identification. The principle reagent used for this extraction was 0.5 per cent ammonium oxalate solution, although ammonium citrate and potassium carbonate solutions were also found to be effective. A subsequent paper will show that these treatments remove a major portion of the pectic material. The pectic acid was identified by comparison with the pectic acid obtained from commercial citrus pectin. Their rotations, titration equivalents, carbon dioxide analyses, and yields of mucic acid were found to be similar.

The committee on pectin nomenclature (1) has defined "pectic substance" as a complex carbohydrate derivative which is characterized by the presence of galacturonic acid units, while "pectic acid" is an hydrolysis product of varying composition. These definitions are rigidly adhered to in this paper.

EXPERIMENTAL PART

Material. The material used for this work was Coker's Super Seven cotton fiber (*Gossypium hirsutum* L. Strain 4) which was grown and ginned under our own direction (7). The ginned cotton was then mechanically ground by the Claremont Waste Manufacturing Company to such a fineness that it would pass through a 40-mesh screen. There was no charring in this process.

Preliminary treatment. The ground cotton was then placed in a Soxhlet extractor, and continually extracted with absolute ethyl alcohol for a period of 24 hours. From the alcohol extract there was obtained upon

[1] This paper was presented before the Cellulose Division of the American Chemical Society meeting at Pittsburgh, Pennsylvania, on September 7, 1936.

evaporation a residue which consisted of a considerable amount of coloring material, and an oily waxy material. Further extraction with alcohol, ether, or benzol did not yield an appreciable amount of this residue. This residue from the alcohol extract is being reserved for future investigation.

After the above described treatment, the cotton was first air-dried and then allowed to stand with distilled water at a temperature of 75° C., for a period of twenty-four hours. About 2.5 liters of water were used per 140 grams of cotton. In later experiments the water extraction was carried out in a Soxhlet extractor over a period of 24 hours with equally good results. The water was removed by filtration and the filtrate clarified by running it through a Sharples continuous centrifuge of the laboratory type. This filtrate was then concentrated under diminished pressure at 40° C. to about one-fifth of its original volume, to which was added twice its volume of 95 per cent alcohol containing 1 per cent hydrochloric acid. A brownish gelatinous precipitate slowly formed which settled to the bottom and was later removed by centrifuging. This precipitate, after purification, gave 5 per cent carbon dioxide by the usual galacturonic acid analysis (4). Further extractions with water yielded very small amounts of this material. The fiber loses about 1 per cent of its original weight by this extraction.

Ash analysis on some samples of this precipitate was as high as 25 per cent. Ash analysis of the extracted cotton averaged 0.19 per cent, while the average ash content of the raw fiber was 0.65 per cent.

Ammonium oxalate treatment. From the cotton, which had undergone the alcohol and water extractions, the main fraction of pectic-like material was obtained by treatment with 0.5 per cent ammonium oxalate solution. One hundred and forty grams of cotton were allowed to stand in 2.5 liters of the 0.5 per cent ammonium oxalate solution for 24 hours at 75° C. After filtration, the solution was clarified, concentrated, and precipitated with acidified alcohol in the same manner as described for the water extract. The main difficulty with this process is that the jelly-like precipitate is usually contaminated with oxalic acid crystals. This difficulty may be partially overcome by careful addition of alcohol to prevent excessive crystallization of oxalic acid. After the third extraction of the cotton fiber with ammonium oxalate, negligible amounts of gelatinous precipitates were obtained.

The use of ammonium citrate as the extractant in the above procedure avoids the difficulty of contaminating the gelatinous precipitate with alchohol-insoluble organic acids.

Purification. The precipitate from the ammonium oxalate extraction was separated by centrifuging, and redissolved in distilled water containing enough ammonia to make the final solution just alkaline. A cloudiness which occurred at this point was removed by clarification in a high

speed continuous centrifuge. The solution was then acidified with hydro-chloric acid, and precipitated by adding an equal volume of 95 per cent alcohol. This purification was repeated four or five times. The final precipitate was then dispersed with distilled water and coagulated with alcohol. This was repeated three or four times. The bulky material was filtered through silk, washed with alcohol and ether, and finally dried in a vacuum desiccator over phosphorus pentoxide. The final precipitate contained 0.4 per cent of ash.

An alternative method of purification by electrodialysis, as described by Emmett (6), was used. The above method of purification was checked on pectic acid from commercial citrus pectin which contained approximately 6 per cent ash. The final ash content was reduced to 0.5 per cent.

Identification. A comparison of the chemical properties of the purified product from the ammonium oxalate extract from cotton with those of the purified pectic acid obtained by hydrolysis of citrus pectin shows them to be of a similar nature. These figures are given in Table I.

TABLE I

COMPARISON OF CITRUS PECTIC ACID WITH THAT FROM COTTON

	Purified pectic acid from citrus pectin	Purified pectic acid from ammonium oxalate extract of cotton
$[\alpha]_D^{28°}$	+240.5° in 0.1 N NaOH where C=0.919%	+225.4° in 0.1 N NaOH where C=0.55%
Titration equivalent	186 1 g. = 53.2 cc. of 0.1 N NaOH	201 1 g. = 49.8 cc. of 0.1 N NaOH
% CO_2	19.9% 19.9%	21.8% 21.7%
Mucic acid yield	47.5%	44.0%

Mucic acid was obtained by oxidation of the pectic materials with 8 per cent hydrobromic acid containing an excess of bromine. The mixture was refluxed until the solution was clear, which took at least three days. The mucic acid crystallized out on standing, and was obtained in a pure state by simply filtering and washing with water; M.P. 209° C. Mixed melting point of the mucic acid from citrus pectin with mucic acid obtained from the product extracted from the cotton fiber showed no depression. Analysis of mucic acid from pectic acid from the cotton fiber follows:

4.236 mg. substance gave 5.314 mg. CO_2 and 1.748 mg. H_2O
5.100 mg. substance gave 6.408 mg. CO_2 and 2.090 mg. H_2O
Theory for $C_6H_{10}O_8$ C, 34.28; H, 4.76
Found   C, 34.21; H, 4.62
C, 34.27; H, 4.59

Yields. Since the purification of the product described above involved considerable loss of material, a direct determination of the pectic acid as calcium pectate (3) was made on an extract of cotton fiber. Due to the interference of the oxalate ion, 0.5 per cent potassium carbonate was used as the extractant. After clarification of the extract in a Sharples centrifuge, the clear filtrate was acidified with acetic acid. An excess of calcium chloride was added, and the gelatinous precipitate of calcium pectate was collected in the Sharples centrifuge. The product was then purified by boiling with distilled water and filtering through silk. This process was repeated until the filtrate contained no chloride ions. The product was washed with alcohol and dried in a vacuum desiccator. The yield from 1100 grams of cotton was 7.5 grams or 0.68 per cent. It contained 12 per cent ash.

Pectic acid in the cotton fiber is apparently present as an insoluble salt of calcium and perhaps other metals. This is inferred by the observation that solutions of salts like ammonium oxalate yield a soluble ammonium or potassium pectate, and leave behind on the fiber insoluble calcium oxalate. Subsequent extraction with dilute hydrochloric acid gave a filtrate from which calcium oxalate was precipitated by the addition of ammonium hydroxide.

The treatments described in this paper did not destroy the fibrous characteristics of the cotton. Unfortunately, apparatus for accurate strength measurements was not available, but the cotton was resistant to disintegration by grinding in a hand mortar.

CONCLUSION

Pectic acid has been extracted from the raw cotton fiber with ammonium oxalate solution.

It was identified by comparison of its rotation, titration equivalent, yield of carbon dioxide, and yield of mucic acid with the same properties of citrus pectic acid.

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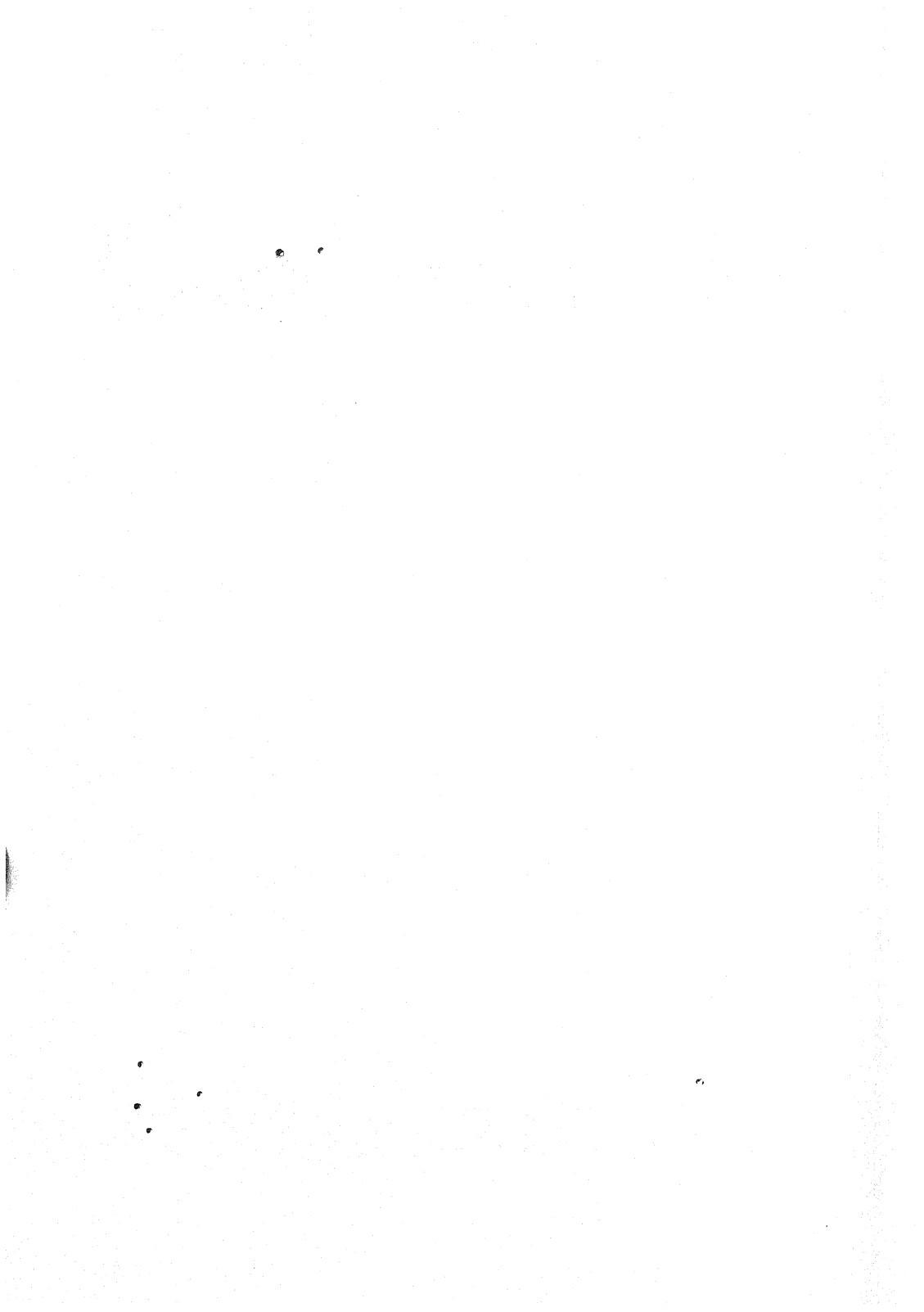
AFTER-RIPENING AT 5° C. FAVORS GERMINATION OF GRAPE SEEDS

FLORENCE FLEMION

An investigation of the conditions required for optimum germination of grape seeds was undertaken following a request from Sicily for information regarding the germination of seeds of our native grapes. The European grape (*Vitis vinifera* L.) which excells all others for its wine qualities can survive *Phylloxera*-infested territory only when grafted on a resistant stock. Since most of our native grapes are resistant to this aphid they are used extensively here and abroad as understock and in breeding experiments.

Grape seeds of the following species and varieties were used: *Vitis aestivalis* Michx. (*V. labrúsca* var. *aestivalis* Regel.) commonly known as summer or pigeon grape, *Vitis bicolor* La Conte (*V. argentifolia* Munson), Concord grape derived from the wild *labrúsca* grape (pure-bred or a cross with either Catawba or *vinifera*) by Ephraim W. Bull of Concord, Massachusetts in September 1849 (4, p. 221) and the Delaware grape which is probably a hybrid of *labrúsca*, *Bourquiniana*, and *vinifera* and was first brought to notice when found thriving near Delaware, Ohio in 1849 (4, p. 232).

The literature available on germination of grape seeds is rather fragmentary considering the great interest in viticulture. Nobbe (5) in 1884 stated that many months are required for full germination. Ramaley (6) in 1902 found that in the common wild grape of the northern United States (*Vitis cordifolia*) some of the seeds germinated within a few weeks, while others did not germinate until the second year. Adams (1) reported that seeds of *Vitis vulpina* which he had planted outside in September 1924 produced a total of 96 per cent germination after 300 days, while seeds planted at the same time and kept for a similar period of time in a greenhouse gave a total germination of only 66 per cent.

The results given in this paper show that the seeds of the four different grapes tested germinate at a warm temperature but that such germination is very sporadic and covers a considerable period of time before a fairly complete germination is obtained. A much greater and more uniform germination is obtained when the seeds mixed in a moist medium are subjected to a period of several months at about 5° C. just prior to being planted in a warm greenhouse (21° C.). Seeds planted out-of-doors in the fall in this latitude are subjected to sufficient low temperature so that germination occurs within a relatively short period of time in the subsequent warm spring.

MATERIALS AND METHODS

The grapes were collected when ripe in the vicinity of Yonkers, New York, except for the variety Delaware which was purchased in the Yonkers fruit market. The seeds were removed from the pulp by the use of a Hobart mixer, washed well, and spread out in thin layers. After thorough drying, they were stored air-dry in containers at room temperature.

The seeds when tested in electrically-controlled ovens were mixed in granulated peat moss[1] which had been moistened and wrung out thoroughly by hand until it crumbled apart readily. The moist medium containing the seeds was placed in bottles and was dampened when necessary by the addition of water. The seeds were kept at various constant temperatures and also at temperatures alternated daily except for Sundays and holidays. In a given daily alternating temperature, as for example 10° and 30° C., the material was kept at the lower temperature (10° C. in this case) for 16 hours and then transferred for 8 hours to the higher temperature (30° C.) for five days every week. On Saturdays, they were held for three hours at the higher temperature and then transferred to the lower temperature and kept there over Sunday for 45 hours. Over an entire week, the seeds had been subjected to a total of 43 hours at the higher temperature and a total of 125 hours at the lower temperature. In the case of a holiday coming during the week, the seeds were held at the lower of the two temperatures for 40 hours.

For plantings in soil the seeds were placed in flats containing a mixture of one-third peat moss, one-third sand, and one-third sod soil. The greenhouse temperature was about 21° C.

RESULTS

Preliminary results. In the first experiment conducted, seeds of the 1934 crop of Concord grape were mixed in moist peat moss on November 6, 1934 and held at the four different temperatures shown in Table I. Over

TABLE I

GERMINATION PERCENTAGE OF CONCORD GRAPE SEEDS WHEN KEPT IN A
MOIST MEDIUM AT VARIOUS TEMPERATURES

	Period at various temp., months	Percentage germination			
		25° C.	10° and 25° C.	15° and 30° C.	5° C.*
Intact seeds	2	0	10	30	——
	4	0	53	41	63
	6	0	55	44	90
Protective tip over radicle removed	2	0	4	12	——
	4	0	30	20	——
	6	0	46	22	32

* Germinations obtained several weeks after transfer to greenhouse.

[1] Purchased from Atkins and Durbrow, New York City.

50 per cent germination was obtained after four months at 10° and 25° C. alternated daily (16 hours at 10° C. and 8 hours at 25° C.) while no germination had occurred even after six months at 25° C. Since grape seeds do not germinate at 5° C., they were planted in the greenhouse after the fourth and sixth month when 63 and 90 per cent germinated within several weeks after planting. The figures in Table I are averages of duplicate lots of 200 intact seeds and averages of duplicates of 50 seeds each in case part of the hard outer coat was removed. Germinations at the daily alternating temperatures (10° and 25° C., and 15° and 30° C.) were scattered over a considerable period of time while the germinations from seeds subjected for four months at 5° C. occurred within several weeks after planting. In every case the intact seeds gave higher germination percentages when compared with the results obtained from the series which at the beginning of the experiment had part of the outer coats removed.

Alternating temperatures. In subsequent experiments other alternating temperatures were tested to determine whether complete germination could be obtained within a short period of time. Grape seeds of Concord, Delaware, *aestivalis*, and *bicolor* were placed in a moist medium at various temperatures alternated daily. The subsequent germinations ranged over a considerable period of time. The highest percentages were obtained with *aestivalis* and the results of one of the tests are shown in Table II. The

TABLE II

EFFECT OF VARIOUS ALTERNATING TEMPERATURES UPON SUBSEQUENT GERMINATION OF SEEDS OF V. AESTIVALIS

Temp. ° C. alternated daily	Percentage germination after various months				
	1	2	3	4	5
1 and 25	0	9	25	39	43
5 and 25	0	10	27	38	42
10 and 25	2	18	38	46	48
15 and 25	2	22	40	63	65
1 and 30	3	32	56	66	68
5 and 30	4	35	56	59	60
10 and 30	4	39	54	71	72
15 and 30	4	36	63	64	68
20 and 30	7	38	60	65	67

germinations recorded in this table are averages of duplicate lots of 200 seeds of the 1934 crop placed at the various temperatures on February 11, 1935. Over 50 per cent of the seeds had germinated at the end of the third month in five of the nine temperatures tested. However, even after five months at the most favorable of the daily alternating temperatures (Table II), the total germination percentage did not exceed the percentage obtained when the seeds were kept at 5° C. for three months and then planted (Table III).

Various low temperatures. Seeds of the 1934 crop of *aestivalis* and Concord were mixed in moist peat moss and placed at four different low temperatures on November 16, 1934. Duplicates of 50 seeds from each lot were planted in the greenhouse after 0, 2.3, 3, and 6.5 months. The subsequent germinations always occurred within several weeks after planting. As seen in Table III the seeds held three months at 5° C. showed optimum germination while the seeds with no previous treatment at low

TABLE III

PERCENTAGE GERMINATION OF GRAPE SEEDS WHEN PLANTED IN THE GREENHOUSE
AFTER VARIOUS MONTHS AT LOW TEMPERATURE

Temp.	Percentage germination after various months							
	aestivalis				Concord			
	0	2.3	3	6.5	0	2.3	3	6.5
1° C.	6	61	75	56	2	1	18	62
5° C.	6	63	75	73	2	10	74	78
Ice box*	6	56	69	74	2	5	74	65
10° C.	6	62	47	50	2	8	65	83

* Temperature in ice box fluctuated from 2° to 8° C.

temperature germinated very poorly. In another test, seeds of the 1934 crop of Concord were planted in the greenhouse after being subjected while in a moist medium for five months to 1°, 5° C. icebox (temperature fluctuated from 2° C. to 8° C.), 10°, 15°, 20°, 25°, and 30° C. The subsequent germinations are illustrated in Figure 1. No germinations occurred at any of the temperatures prior to planting except at 30° C. where 4 per cent germinated before planting and 5 per cent germinated while in the greenhouse. The results in Figure 1 show very clearly the efficacy of a pretreatment at low temperature.

Room temperature storage. Other tests were made with seeds of the 1935 crop of *bicolor*, Concord, and Delaware at the time the seeds were collected and at various intervals up to twelve months from the time of harvest. The seeds (lots of 200 each) were planted in the greenhouse after being subjected while in a moist medium to 5° C. for 0, 3, and 4.5 months. Very poor germination results were obtained when the control seeds were planted directly in the greenhouse (Table IV), while high percentages resulted when the seeds had been pretreated at 5° C. In the case of Delaware grape, fresh seeds as well as those previously stored germinated equally well after a period of three months at 5° C. Seeds of *bicolor* and Concord required a longer pretreatment at low temperature when fresh or old as compared with seeds from the same lot after air-dry storage at room temperature for several months prior to the period at 5° C. Hence periods of dry storage were found which decreased the time required at low temperature for optimum germination, but no amount of previous dry
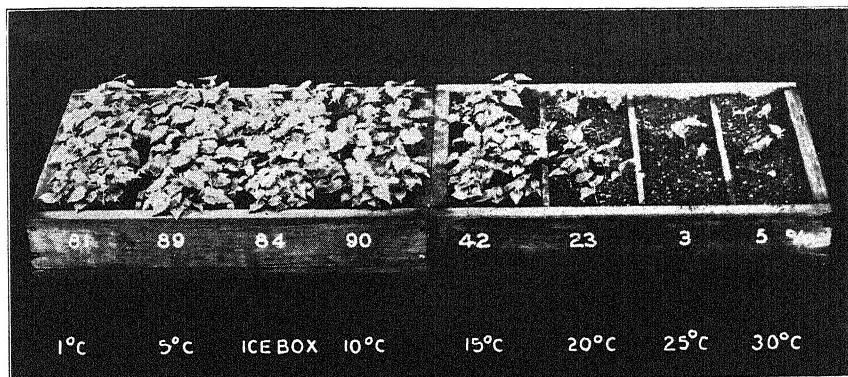
FIGURE 1. Concord grape seedlings (percentage germination) produced in a 'warm greenhouse when lots of 100 seeds each of the 1934 crop were pretreated in a moist medium for five months at the various temperatures. Photographed on March 29, 1935 six weeks after planting.
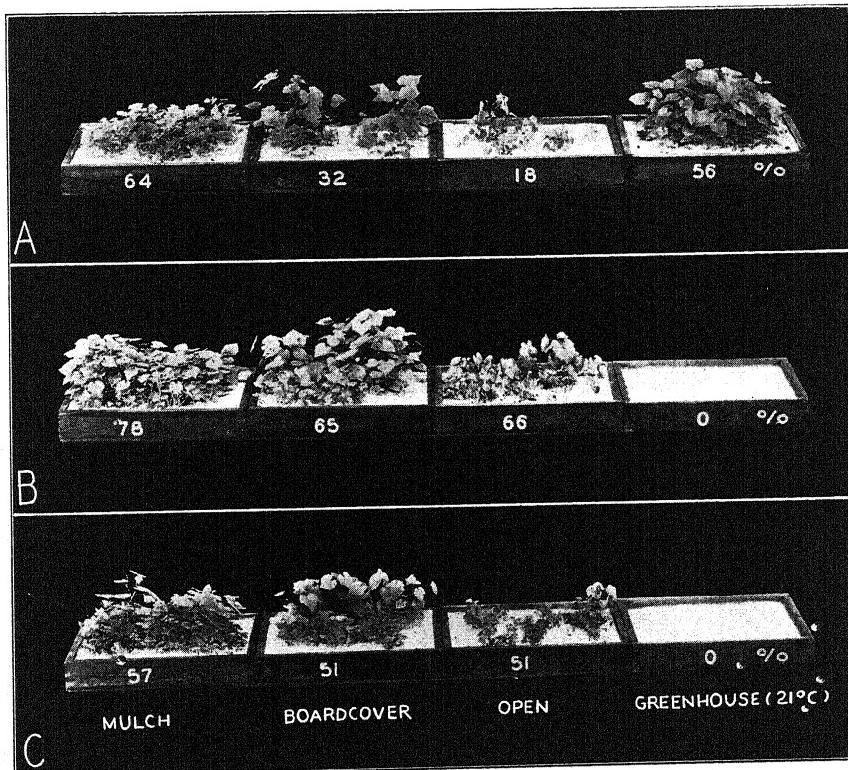
FIGURE 2. Grape seedlings photographed August 9, 1935. Lots of 200 seeds each of the 1934 crop of (A) *aestivalis*, (B) Concord, and (C) Delaware planted on October 1, 1934 and kept out-of-doors at various conditions.

TABLE IV

EFFECT OF VARIOUS PERIODS AT 5° C. UPON SUBSEQUENT GERMINATION OF GRAPE SEEDS

Period of dry storage at room temperature, months	Percentage germination when planted in greenhouse after various months at 5° C.								
	bicolor			Concord			Delaware		
	0	3	4.5	0	3	4.5	0	3	4.5
0	0	9	49	0	22	73	4	63	76
1.5	0	47	70	0	66	82	0	76	78
3	2	56	76	14	63	83	12	—	—
4.5	0	—	69	0	70	68	4	—	—
6	8	66	62	0	38	72	12	66	70
7	10	44	50	0	17	42	4	56	72
12	1	37	61	0	23	56	3	44	53

storage entirely eliminated the necessity of this pretreatment. After about 6 months of dry storage at room temperature the seeds begin to slowly lose their vitality and are then favored by longer pretreatments such as 4.5 months at 5° C. (Table IV).

TABLE V

SEEDLING PRODUCTION OF GRAPE SEEDS WHEN PLANTED OUT-OF-DOORS IN COLD FRAMES

Variety	Percentage germination in the spring							
	Mulched cold frame		Frame with board cover		Open cold frame		Control (21° C. greenhouse)	
	1934 crop	1935 crop	1934 crop	1935 crop	1934 crop	1935 crop	1934 crop	1935 crop
aestivalis	65	—	36	—	16	—	56	—
bicolor	—	27	—	14	—	5	—	0
Delaware	56	60	51	66	46	54	0	4
Concord	79	71	70	70	65	59	0	0

Cold frames. Seeds of the 1934 and 1935 crops were planted in flats in the fall and placed in cold frames. Three conditions out-of-doors were used—cold frames without cover, cold frames with a board cover, and board-covered cold frames within which the flats containing the seeds were mulched (covered with dry leaves). The controls were kept in a warm greenhouse at about 21° C. Plantings of the 1934 crop were made on October 1, 1934 and those of the 1935 crop were made on September 30, 1935. Several counts were made the subsequent springs. The results appear in Table V. The percentages given for the 1934 crop are averages of triplicate lots of 200 seeds, while the figures for the 1935 crop are averages of duplicate lots of 200 seeds. The seeds of Delaware and Concord germinated very poorly when kept in the warm greenhouse but gave a high seedling production in the flats subjected to the cold out-of-doors during the winter months. In the case of *aestivalis* seeds the high germination of 56

per cent obtained in the greenhouse was prolonged over the winter months while 65 per cent germination was obtained within a few weeks after having been in the mulched frame over winter. Some of the seeds in the board cover and open frames were probably killed by the rather severe changes in temperature in these frames. The mulched condition maintains a more even temperature throughout the winter. The illustrations in Figure 2 (one set of the triplicates averaged in Table V) show the development and percentage survival of the seedlings of the 1934 planting of seeds of *aestivalis*, Concord, and Delaware when photographed the following summer.

In the discussion of Table IV it was pointed out that seeds of *bicolor* do not respond so readily if subjected to low temperature when freshly-harvested. The seeds of *bicolor* and *aestivalis* were planted and placed in the cold frames only five and ten days respectively after being collected and cleaned and this may account for the low germination percentages shown in Table V for these two species. In this connection it is interesting to note that the Delaware grape seeds of the 1935 crop which were planted 19 days after the time of harvest produced consistently higher germination percentages than the seeds obtained (Table V) from the 1934 crop planted only five days after harvest. The 1934 and 1935 crops of seeds of Concord grape were planted 11 and 20 days respectively after harvest.

DISCUSSION

Grape seeds can be germinated readily at various temperatures but the optimum number of seedlings can be obtained within a period of a few weeks when the seeds are given a pretreatment of several months at 5° C. just prior to planting. The seeds collected when ripe should be freed of the pulp, dried, and then stored at room temperature. Since the most effective period of air-dry storage was found to be 3 to 6 months, the seeds should therefore be mixed in moist peat moss in mid-winter and placed at about 5° C. Very little space is required since a great number of seeds can be mixed with a relatively small amount of moist peat moss. This material placed in a container and covered with cheesecloth needs no special attention while at the low temperature except that it be watered about twice a month. After adding a little water the whole mass should be thoroughly mixed. After 3 to 4 months in this condition, the seeds— easily freed of the peat by washing through a sieve—can be planted in the spring either in the greenhouse or out-of-doors. Anyone having available a cool cellar, an electric refrigerator, or an icebox running at about 5° C. can use this method. Another method is to plant the seeds in the fall out-of-doors in cold frames, but the total yield is not as great as when the above described low temperature pretreatment is employed. In addition, the cold frames involve space and added labor.

Some seedlings can also be obtained by planting the seeds directly in a warm greenhouse. The germination percentage is usually quite low and considerable time elapses before a number of seedlings is obtained. Another way to obtain seedlings is to keep the seeds while in a moist medium at certain daily alternating temperatures. The peat moss dries out rapidly at these temperatures and should be watered frequently, at least once and often twice weekly. In addition, the germinated seeds as they appear must be picked out and planted. There is considerably less trouble involved when the material is subjected to 5° C. because water added only every second or third week is sufficient. Germinations rarely occur at this low temperature and all of the seedlings appear within a few weeks after the seeds are planted in a warm place. Moreover, a higher total germination results when the seeds are subjected to this cold treatment.

Chauveaud (2) stated that by removing part of the seed coat of *V. rupestris*—the protective tip over the hypocotyl—the seeds germinated within ten days at 27° C., while seeds at this temperature with only split coats did not germinate even after two months. Such experiments in our laboratory gave consistently lower results than intact seeds under similar conditions. The embryos were probably injured during the removal process.

Since seedlings were obtained with or without a pretreatment at low temperature, they were examined rather frequently to determine differences in rate of growth, etc. In some of the rosaceous forms (3) seeds, which normally require a pretreatment at 5° C. before germination occurs, when forced to germinate at 25° C., produce seedlings which manifest dwarfish characteristics. As far as could be ascertained all the grape seedlings observed were normal.

The method of a pretreatment at 5° C. as suggested in this paper should be of considerable assistance in the germination of grape seeds either from the standpoint of developing new hybrids or of producing a maximum number of seedlings at a given time for understock.

SUMMARY

1. Optimum germination results were obtained when grape seeds (*Vitis aestivalis*, *V. bicolor*, Concord, and Delaware) were pretreated for three to four and one-half months in a moist medium at about 5° C. and then planted in a warm place.

2. Seeds also germinated at various temperatures alternated daily, but the germination extended over a considerable period of time.

3. Removing part of the seed coat over the radicle reduced the germination.

4. Seeds of *bicolor* and Concord stored air-dry for several months respond to shorter periods at low temperature than do older or freshly-harvested seeds.

5. A good seedling production can be obtained in the spring by planting the seeds out-of-doors in the fall in a mulched cold frame.

6. Even though seeds germinate at a warm temperature the total germination percentage can be increased considerably by a period in a moist medium at about 5° C. prior to planting.

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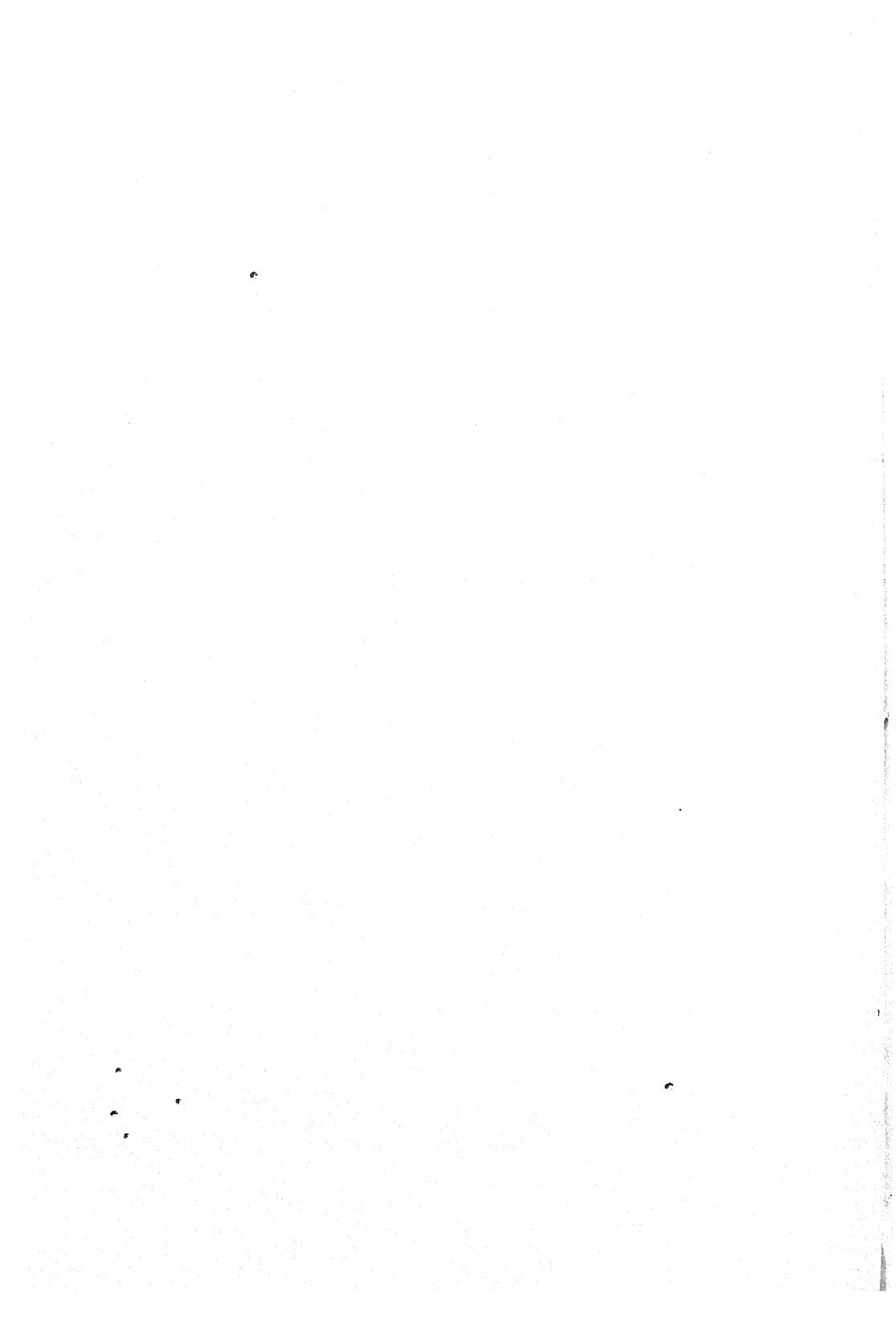
FACTORS INFLUENCING THE DEVELOPMENT OF ASCORBIC ACID AND GLUTATHIONE IN POTATO TUBERS FOLLOWING TREATMENT WITH ETHYLENE CHLORHYDRIN[1]

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Potatoes are an important source of ascorbic acid (vitamin C) in the diet of many people. Although they contain less ascorbic acid than a number of other foods such as the juice of citrus fruits and tomatoes, they are eaten so frequently and in such quantities that large amounts of ascorbic acid are obtained from them. New potatoes contain more ascorbic acid than old potatoes, since ascorbic acid decreases in the tubers during storage. At the end of winter they are low in ascorbic acid and this is the time that fresh vegetables are most likely to be limited in the diet. A method that could be used to increase the ascorbic acid content of old potato tubers might prove useful for increasing their food value. Any factors that change the ascorbic acid content are interesting from this standpoint in addition to their theoretical significance.

In previous papers (5, 6) it has been shown that treatment of potato tubers with ethylene chlorhydrin and other chemicals increases the glutathione content. It is customary to take ascorbic acid into consideration in dealing with glutathione because both react with iodine in acid solution and both play a part in the oxidation-reduction processes of cells. Because of this, a number of ascorbic acid determinations were made in connection with the work on glutathione, but these were not published, since no change was observed following treatment with ethylene chlorhydrin. Recently Pett (9) has presented data showing that ascorbic acid increased following treatment with ethylene chlorhydrin. A perusal of the unpublished determinations of ascorbic acid showed that they were all made on uncut tubers, while Pett used cut tubers and subsequent work has shown that this explains the apparent discrepancy. Since cutting had such a marked effect on the behavior of ascorbic acid and, as later experiments showed, of glutathione, it was decided to investigate the effect of this and other factors on the development of ascorbic acid and glutathione in potato tubers following treatment with ethylene chlorhydrin.

TREATMENTS AND SOURCES OF TUBERS

The chemical treatments were made according to the methods of Denny (1). Unless otherwise stated, vapor treatments of whole tubers were used. A 3.5 liter container was half filled with the tubers, 3 or 4 cc.

of 40 per cent ethylene chlorhydrin were poured on cotton above the tubers, and the container sealed with plasticine. The check tubers were placed in a similar closed container. In the cases where dip treatments were made, cut pieces were dipped in a solution of ethylene chlorhydrin, 40 cc. of 40 per cent per liter, the solution drained off, and the pieces kept in a closed container. The check tubers were dipped in water. The duration of all of the treatments was 24 hours.

Various lots of tubers were used for the experiments. One lot was second crop Irish Cobblers bought in New Jersey early in November. Small, rather immature tubers grown in the greenhouse were used for some of the experiments. However, most of the new tubers were bought locally, but were southern-grown. The Bliss Triumph tubers came from Cuba, and from Belle Glade and Princeton, Florida. The latter lot was grown by Mr. John W. Campbell who verified the variety. One lot of Spaulding Rose, presumably from Florida, was used. Several lots of old Green Mountain tubers and one lot of old Irish Cobblers were also used. These old tubers were well out of their rest period. The use of so many varieties and sources of tubers was necessitated by the difficulty of getting suitable material during the winter months. As a result, however, certain conclusions are not limited to a particular variety or source of tuber.

DETERMINATION OF GLUTATHIONE

Glutathione was determined by a modification of the sulphur reduction method of Guthrie and Wilcoxon (6). It was desirable to modify this method so that ascorbic acid could be determined on the same extract. The original method called for dropping the tissue into boiling water, cooling, and extracting with alcohol. This extract is not suited to analyses for ascorbic acid, since low values are obtained. However, the conclusion of Pett (9) that low glutathione values are found could not be verified, since the non-ascorbic acid iodine reducing substances, called glutathione by Pett, were no lower than found with metaphosphoric acid extraction. It was found, however, that the metaphosphoric acid extract could be used for the determination of glutathione by the sulphur reduction method, and since it is somewhat more convenient than the alcohol extraction and also may be used for the determination of ascorbic acid, as shown by Fujita and Iwatake (4), it was used in the present investigation.

The tubers or cut pieces were washed and the callus was cut from the cut pieces, but in no case was the peel removed. In some of the later experiments, after it was found that the cut surface played an important part in some of the changes observed, the callus was not removed. This is indicated in the text or tables.

The extraction was made by placing 50 g. of the tissue, taking care to obtain a representative sample, into a large mortar containing 50 cc. of

freshly prepared 5 per cent metaphosphoric acid and 10 g. of washed quartz sand. The tissue was then crushed and ground until fine. An additional 50 cc. of metaphosphoric acid and 50 cc. of water were added. The suspension was poured into centrifuge bottles and allowed to stand 20 minutes with frequent shaking. It was then centrifuged and the supernatant liquid filtered through absorbent cotton.

Since some oxidase seemed to remain in the metaphosphoric acid extract, it was necessary to add KCN before neutralization of the extract for the determination of glutathione. The procedure used for most of the determinations was to add 3 cc. of 1 per cent KCN to 60 cc. of the extract and adjust to pH 6.6 to 6.8 with 20 per cent NaOH. About 2 cc. were required. The addition of a few drops of bromothymol blue aided in the adjustment of pH. After the addition of the KCN the solutions should not be measured with a pipette, since HCN is present in the vapors above the solution. Careful measurement in a graduated cylinder was found to be sufficiently accurate and should lead to less than 2 per cent error.

After adjustment of the pH, 50 cc. portions of the extracts were measured at once into Van Slyke-Cullen aeration tubes, 10 drops of paraffin oil and 2 cc. of a saturated solution of sulphur in absolute alcohol added and the solutions aerated with nitrogen into tubes containing 25 cc. of 2 per cent zinc acetate for four hours. At the end of this period, 5 cc. of para-amino-dimethylaniline hydrochloride, 100 mg. per 100 cc. of 20 per cent HCl, and 5 cc. of $M/50$ $FeCl_3 \cdot 6H_2O$ in 1:9 HCl were added to the receiver. Methylene blue developed proportionally to the amount of H_2S absorbed by the zinc acetate. The solutions were allowed to stand overnight so that any zinc cyanide would go into solution. The methylene blue solutions were then made to volume, usually 50 cc., and compared in a colorimeter with standards prepared from known amounts of hydrogen sulphide.

The standards were prepared from a N/1000 solution of H_2S in dilute NaOH. This was prepared by dilution from a concentrated solution made by bubbling H_2S through 100 cc. of water and 10 cc. of N/1 NaOH for 5 minutes, adding 10 cc. more of the NaOH and diluting to 1.5 liter. The concentration of this solution was determined by iodometric titration using N/50 iodine and N/100 $Na_2S_2O_3$ and the amount required to make a N/1000 solution diluted to one liter. The series of standards usually used were made by adding 1, 2, 5, and 10 cc. of the N/1000 solution to 25 cc. of 2 per cent zinc acetate in 50 cc. flasks, adding 5 cc. of para-amino-dimethylaniline hydrochloride and 5 cc. of ferric chloride and making to volume.

Since in the above procedure, glutathione did not yield the theoretical amount of H_2S called for by the reaction, $2GSH + S \rightarrow H_2S + GSSG$, it was necessary to determine the amounts of hydrogen sulphide obtained

from known amounts of glutathione using the same procedure as in the determinations. For this purpose, known amounts of glutathione were added to metaphosphoric acid solutions containing the required amount of KCN and adjusted to pH 6.6 to 6.8, or to extracts of potato tissue in which reduced glutathione had been removed by adjusting to pH 6.6 to 6.8, adding a saturated solution of sulphur in alcohol and aerating with nitrogen for four hours. Potassium cyanide was then added, the pH readjusted and known amounts of glutathione added to 50 cc. portions just before adding the usual 2 cc. of sulphur in alcohol and aerating. In such an experiment, 1, 2, 3, 4, 6, and 8 mg. of glutathione yielded 1.7, 3.9, 6.8, 10.3, 17.1, and 25.5 cc. of $N/1000$ H_2S respectively from metaphosphoric acid solutions and 1.9, 4.6, 7.0, 9.6, 16.4, and 23.1 cc. $N/1000$ H_2S respectively from potato extract prepared as described above. It will be seen that with the procedure used, the yield from potato extracts was essentially the same as from metaphosphoric acid solutions.

Since the procedure for the sulphur reduction method was under investigation while the experiments reported in this paper were being made, various modifications of the procedure were used. However, in no case did these modifications affect the yield of H_2S over 10 per cent, with the exception of a few early determinations made when the importance of adding KCN was not known. In all cases where the procedure was modified, a recovery curve for known amounts of glutathione made by the modified procedure was used in the calculations.

In obtaining the values reported, which are in terms of cc. $N/100$ per 100 g. tissue, it was assumed that volume obtained by adding 150 cc. of fluid to 50 g. of fresh tissue, composed mostly of water, was 200 cc. The 60 cc. aliquot of the extract used for the glutathione determinations, therefore, represented 15 g. The dilution of this by the addition of 3 cc. of 1 per cent KCN and 2 cc. of 20 per cent NaOH makes the amount of tissue represented by 50 cc. used in the aeration 11.54 g.

DETERMINATION OF ASCORBIC ACID AND OTHER REDUCING SUBSTANCES

A 25 cc. aliquot of the metaphosphoric acid extract, which was assumed to represent 6.25 g. tissue, was titrated with 2,6-dichlorophenolindophenol, using a solution prepared by grinding 200 mg. of the dye with water in a mortar, making up to 200 cc. and filtering. The dye was made fresh every few days and was standardized against a weighed portion of ascorbic acid not over two days prior to use. From this titration the ascorbic acid content was calculated in terms of cc. $N/100$ per 100 g. tissue.

Another portion of the filtrate was titrated with $N/100$ iodine in $N/20$ KI. After calculating the cc. $N/100$ iodine per 100 g. of tissue, the value

for ascorbic acid was subtracted from this. The difference includes gluta-
thione and any other iodine-reducing substance present in the tissue.
Since glutathione values obtained by the sulphur reduction method are
much lower, it is assumed that iodine-reducing substances other than
ascorbic acid and glutathione are present in the tissue. This value obtained
by difference is, therefore, called non-ascorbic acid reducing substances.

It is possible by subtracting the glutathione values as well as the
ascorbic acid values from the iodine values to obtain a figure which should
represent iodine-reducing substances other than ascorbic acid or gluta-
thione. This value is not very accurate since it is affected by the error of
three determinations. It was calculated wherever possible but is not re-
ported in the tables, since in general it showed the same changes as
glutathione.

EFFECT OF CUTTING

Since the explanation for the apparent discrepancy between the results
of Pett (9) and some unpublished analyses for ascorbic acid was found to
be due to Pett's treating cut pieces which were subsequently planted,
while the unpublished data were for uncut tubers, an extensive study of
the effect of cutting and planting was made. The results are shown in
Table I. In the first experiment in which freshly-harvested Irish Cobblers
were used, the whole tubers showed no increase in ascorbic acid due to
treatment with ethylene chlorhydrin. However, the same tubers when cut
into pieces and planted showed a definitely higher ascorbic acid content
on the sixth day in the treated sample. This was also true of pieces that
were cut just before treatment by the dip method and then planted. The
data, however, show that no true increase in ascorbic acid took place in
the treated sample, but that the ascorbic acid content of the treated sample
remained rather constant while the ascorbic acid content of the check
sample decreased. The second experiment of Table I was made with the
same tubers after about 3 months' storage. In this case the ascorbic acid
content of the untreated whole tubers had fallen to about one-half of that
found in the freshly-harvested tubers. In this case also, no significant
increase in ascorbic acid due to ethylene chlorhydrin treatment took place
in the whole tubers, but in the cut tubers a large increase took place. This
was a true increase and not due to a decrease in the check tubers.

It seems that the ethylene chlorhydrin treatment does not increase the
ascorbic acid content beyond a certain level, and hence if the ascorbic acid
content is already high, serves only to maintain this high level; while if the
ascorbic acid content is low, the treatment increases the ascorbic acid con-
tent above this low level. In order to show this more clearly, pieces of the
second crop Irish Cobbler tubers were planted for one week. At the end of
this time the ascorbic acid content had fallen to 11.2 cc. N/100 per 100 g.
tissue. The callus that had formed at the cut surface was removed in a

TABLE I

EFFECT OF CUTTING ON THE DEVELOPMENT OF GLUTATHIONE AND ASCORBIC ACID IN POTATO TUBERS FOLLOWING TREATMENT WITH ETHYLENE CHLORHYDRIN

Tubers used	Method of treatment	Condition of tubers after treatment	Days after start of treat.	Ascorbic acid Treated	Ascorbic acid Check	Non-ascorbic acid reducing substances Treated	Non-ascorbic acid reducing substances Check	Glutathione Treated	Glutathione Check
Irish Cobbler, New Jersey 2nd crop, freshly-harvested	Vapor	Whole	3	20.1	19.5	16.7	7.7	11.7	2.7
			6	19.3	19.9	22.7	7.3		
			10	20.9	19.2	28.7	7.2		
			13	19.4	17.7	29.4	6.3		
		Cut and planted	3	23.0	18.2	21.0	7.4	12.3	2.3
			6	19.9	13.4	26.5	5.8		
			10	22.1	11.1	29.1	6.5		
			13	21.7	9.7	27.1	6.3		
	Dip, cut pieces	Cut and planted	3	20.1	18.2	18.3	6.6	13.2	2.3
			6	25.1	13.4	26.1	5.8		
			10	24.0	10.5	29.6	6.3		
			13	21.2	10.3	23.6	6.5		
Same as above, after about 3 mo. storage	Vapor	Whole	3	10.3	10.5	10.5	7.9	5.5	4.0
			7	11.7	10.6	13.9	7.8	8.9	3.3
		Cut and planted	3	17.2	12.5	20.4	7.5	8.6	3.0
			7	19.7	12.4	26.4	6.8	16.8	3.5
Irish Cobbler, old tubers	Vapor	Whole	2	9.5	10.0	10.5	8.4	2.0	4.4
			3	11.2	11.2	7.2	8.0	2.6	3.0
			5	10.6	10.6	9.4	9.4	4.3	4.4
			7	13.2	10.6	12.4	7.8	5.7	4.7
		Cut and planted	2	11.9	10.8	8.1	7.6	2.8	3.2
			3	13.9	11.2	12.2	8.0	4.9	3.8
			5	15.9	11.0	18.5	7.4	8.2	2.4
			7	15.4	11.9	15.0	7.3	9.5	4.8
	Dip, cut pieces	Cut and planted	2	11.7	11.2	5.9	7.5	2.4	2.7
			3	12.5	11.2	9.1	7.2	3.9	3.3
			5	15.9	11.0	16.1	7.4	9.2	3.1
			7	18.9	11.4	17.9	7.0	11.2	4.0
Irish Cobbler, old tubers	Vapor	Whole	3	10.6	9.3	8.8	10.1	4.4	4.2
			8	13.2	9.1	10.0	10.9	5.5	4.9
		Cut and planted	3	17.8	11.0	32.1	8.3	11.7	4.0
			8	20.7	11.2	29.7	7.2	15.8	3.2
Bliss Triumph from Florida, new	Vapor	Whole	3	28.6	32.6	5.0	7.4	2.4	3.5
			7	25.6	30.0	6.4	9.2	2.5	5.1
		Cut, moist chamber	3	33.1	28.3	11.7	8.5	5.9	3.1
			7	31.1	18.4	16.1	7.2	9.7	3.7

thin layer and half of the pieces were treated with ethylene chlorhydrin by the dip method. Four days later the treated sample had an ascorbic acid content of 20.4 while the check sample that had been dipped in water had an ascorbic acid content of 11.9.

In the third and fourth experiments of Table I, old Irish Cobblers were used. These were well out of their rest period and had a low ascorbic acid content. An increase in ascorbic acid took place in the cut pieces and also to a smaller extent and more slowly in the whole tubers. Later experiments show that a large increase in ascorbic acid can be obtained in whole, non-dormant tubers after about 20 days. However, the ascorbic acid change takes place in three to six days in cut pieces.

The last experiment of Table I was made with new Bliss Triumph tubers from Florida. The ascorbic acid content of these tubers was high. The treatment produced no increase in the whole tubers, but the cut pieces of the treated sample maintained a high ascorbic acid content, while the pieces of the check sample rapidly lost ascorbic acid. The cut pieces in this experiment were kept in moist chambers instead of being planted. This shows that the effect observed is due to cutting and that planting is not necessary. Additional evidence for this will be found in later tables.

The difference in the ascorbic acid due to treatment with ethylene chlorhydrin is not due to a change of reduced ascorbic acid to its oxidized form in the planted check tubers, since analysis of the juice and of the trichloracetic acid extract of treated and check cut pieces by the method of Emmerie and van Eekelen (3) which includes both oxidized and reduced ascorbic acid showed the treated sample to be much higher in ascorbic acid.

Table I also shows large increases in glutathione due to treatment with ethylene chlorhydrin such as have been reported previously (5). The freshly-harvested Irish Cobbler whole tubers showed a larger and quicker increase than the old tubers. In general, freshly-harvested tubers give larger and quicker response to chemical treatment. It will also be noted that glutathione behaved similarly to ascorbic acid in being affected by cutting, since a larger and more prompt increase in glutathione was observed in the cut pieces. With respect to glutathione the Bliss Triumph tubers from Florida behaved exceptionally, since no increase was observed in the whole tubers up to seven days after treatment. This also proved true of another lot of Bliss Triumph tubers from Florida and also of a lot from Cuba. However, in later experiments a large increase in glutathione was observed in these tubers in a sample taken 11 days after treatment and in another sample taken 27 days after treatment. The glutathione increase, therefore, takes place even in these southern-grown Bliss Triumph whole tubers, but very much more slowly than in most tubers. There is no definite answer as yet as to whether this delayed increase in glutathione is a varietal difference or due to the conditions under

which the tubers were grown. Some immature, second crop, Bliss Triumph tubers grown in Yonkers showed a prompt increase in glutathione.

The same conclusions concerning glutathione can be drawn from the data in Table I even though the value for non-ascorbic acid reducing substances is considered to be glutathione. Since the sulphur reduction method, which is much more specific for glutathione than the iodine titration, shows lower values, iodine-reducing substances other than glutathione are present in potato tubers. These, however, seem to parallel glutathione in the changes that occur due to treatment with ethylene chlorhydrin.

Since cutting had such a marked effect on the development of glutathione and ascorbic acid in treated tubers, the question arose as to whether the effect of cutting was due to the admission of extra oxygen or to the occurrence of some chemical change in the tuber that aided the development of glutathione. If the tubers were cut previous to treating and a callus allowed to form, they should show a large increase in ascorbic acid and glutathione following treatment if some permanent change had resulted from the cutting. However, if the admission of oxygen was an important factor, the newly formed callus might hinder its entrance as much as the peel and the cut pieces would act like whole tubers. Table II shows that cutting, to be effective, must follow or precede the treatment by only

TABLE II

EFFECT OF CUTTING ONE WEEK BEFORE TREATMENT ON THE DEVELOPMENT OF GLUTATHIONE AND ASCORBIC ACID IN POTATO TUBERS

Condition of tubers	Days after start of treatment	Ascorbic acid		Non-ascorbic acid reducing substances		Glutathione	
		Treated	Check	Treated	Check	Treated	Check
Cut 1 week before treatment	3	14.2	15.0	6.6	6.6	2.5	3.1
	7	12.4	13.9	14.8	7.7	9.2	3.2
Cut after treatment	3	26.0	18.4	18.8	5.6	10.3	3.4
	7	23.3	16.2	19.1	6.2	12.8	3.6
Whole	3	18.4	17.8	8.0	7.0	4.8	3.4
	7	16.2	17.2	11.8	6.8	6.8	3.2

a short interval. The data show that no increase in ascorbic acid took place in either the whole tubers or in the pieces with the old cut surface, but that pieces cut from the same whole tuber immediately after treatment showed a large increase in ascorbic acid. The behavior of glutathione is similar, the increase in the whole tubers and in the cut pieces with the old cut surface was of about the same magnitude, but was smaller and occurred later than the increase in the freshly cut pieces. It appears from these results that the cut surface has changed within one week in such a way as to act like the peel, so that the pieces with the old cut surface act very much like whole tubers.

In some previous experiments (7) it was observed that butyl bromide treatments did not increase the glutathione content of whole potato tubers. It was pointed out that this was an exception to the rule (5) that treatments which increase the pH also increase the glutathione content since a large increase in pH was observed with butyl bromide treatments. Since cutting has such a marked effect on the development of glutathione, and since the correlation between the pH increase and the increase in glutathione was established using cut pieces, it was thought that if tubers were cut and planted following treatment with butyl bromide an increase in glutathione might occur. Table III shows that this proved to be the case and that the glutathione increase observed in the cut pieces was of the same order of magnitude as observed with ethylene chlorhydrin treatments. An apparent exception to the correlation between an increase in pH and an increase in glutathione has, therefore, been explained.

TABLE III

EFFECT OF CUTTING ON THE DEVELOPMENT OF GLUTATHIONE AND ASCORBIC ACID FOLLOWING TREATMENT WITH BUTYL BROMIDE

Tubers used	Condition of tubers after treatment	Days after start of treatment	Ascorbic acid		Non-ascorbic acid reducing substances		Glutathione	
			Treated*	Check	Treated*	Check	Treated*	Check
Spaulding Rose, new	Cut and planted	7	25.1	17.2	21.1	6.0	12.0	2.7
Bliss Triumph	Whole Cut and planted	5 5	18.6 23.2	14.2 16.2	7.8 16.8	9.0 5.4	4.0 10.9	4.1 2.5

* 0.5 cc. of butyl bromide per 3.5 liter container one-half full of tubers.

The marked effect of cutting on the development of glutathione and ascorbic acid in potato tubers following treatment with ethylene chlorhydrin led to experiments in which the cut surface, which had not previously been included in the samples, was analyzed separately. The results of these experiments are given in Table IV. It was found that the changes started a day or so sooner in the cut surface. In the first experiment Spaulding Rose tubers that had been stored one month after purchasing in the local market were used. The ascorbic acid was much higher in the cut surface of both the treated and check sample, than in the inside of the pieces up to the fourth day. A marked drop took place in the ascorbic acid content of the surface of the check sample on the seventh day. In the cut surface, the treated sample was significantly higher in ascorbic acid than the check sample on the third day, but the change inside of the piece appears to start about the fourth day. In the cut surface the glutathione was higher than the check sample on the second day and reached

TABLE IV

DEVELOPMENT OF GLUTATHIONE AND ASCORBIC ACID IN THE CUT SURFACE COMPARED WITH
THE REST OF THE PIECE FOLLOWING TREATMENT WITH ETHYLENE CHLORHYDRIN

Tubers used	Part used	Days after start of treatment	Ascorbic acid		Non-ascorbic acid reducing substances		Glutathione	
			Treated	Check	Treated	Check	Treated	Check
Spaulding Rose stored 1 month	Cut surface	2	23.6	20.3	16.4	8.5	4.8	2.7
		3	32.0	26.0	16.8	6.8	9.4	2.8
		4	33.8	26.0	15.0	6.8	9.0	—
		7	30.1	9.7	15.5	5.5	8.9	1.8
	Inside	2	16.2	15.4	11.0	7.0	3.0	2.8
		3	17.1	15.4	11.7	7.0	6.2	2.8
		4	19.9	16.7	15.3	6.5	9.0	2.8
		7	22.3	14.9	17.7	6.7	10.4	2.5
Bliss Triumph stored 1 month	Cut surface	2	18.0	16.3	6.8	3.7	1.7	1.3
		3	34.8	18.8	22.8	6.8	9.0	1.7
		6	29.3	8.9	25.1	4.7	14.5	2.5
		8	21.8	4.9	25.4	4.7	12.7	—
	Inside	2	11.4	9.4	6.2	7.4	1.6	2.1
		3	13.1	11.5	14.1	5.3	3.2	1.7
		6	21.2	10.4	28.4	4.8	17.5	2.0
		8	22.2	9.0	28.2	4.6	16.2	1.8
	Entire piece	2	12.2	10.2	6.3	7.0	1.6	2.0
		3	15.2	12.3	14.9	5.5	3.6	1.7
		6	22.3	10.2	28.0	4.8	17.1	2.1
		8	22.2	8.6	27.8	4.8	15.8	—
	Uncut tuber	2	10.2	9.4	5.8	8.2	1.6	3.7
		3	8.6	8.6	6.6	7.4	1.4	2.8
		6	8.9	8.1	7.9	5.5	2.8	2.5

its maximum on the third day. Inside of the piece the glutathione increased on the third day and reached its maximum on about the fourth day.

In the second experiment Bliss Triumph tubers were used. These were probably from Florida and had been stored one month. The results were similar to those of the first experiment. Ascorbic acid was higher in the cut surface than inside of the check on the second and third day, but then decreased so that on the eighth day it was lower than inside of the piece. The cut surface of the treated sample was much higher than the cut surface of the check sample on the third day, but a large difference between treated and check was not noted inside of the piece until the sixth day. Glutathione also increased somewhat sooner in the cut surface. In this experiment data for the entire piece were calculated and analyses also made on some of the whole tubers from which the pieces were cut after treatment. From the values for the check whole tubers, it will be seen that the increases in glutathione and ascorbic acid were true increases and not due to a decrease in the check sample. A comparison of the entire

cut piece with the whole tuber shows the usual effect of cutting. The untreated, entire cut pieces are also considerably higher in ascorbic acid than the untreated whole tubers, especially on the third day.

EFFECT OF OXYGEN

A likely explanation for the favorable effect of cutting on the increase in glutathione and ascorbic acid following ethylene chlorhydrin treat-

TABLE V

EFFECT OF CUTTING AND OF EXCLUDING AIR FROM THE CUT SURFACE WITH PARAFFIN ON THE DEVELOPMENT OF GLUTATHIONE AND ASCORBIC ACID IN POTATO TUBERS

Condition of tubers after treatment	Days after start of treatment	Ascorbic acid		Non-ascorbic acid reducing substances		Glutathione	
		Treated	Check	Treated	Check	Treated	Check
Cut and planted	4	20.0	13.7	25.6	7.1	11.4	4.7
	7	21.2	14.6	22.0	7.0	12.8	2.4
Cut and stored in air	4	18.7		18.1		10.6	
	7	24.6		21.8		10.6	
Cut and surface paraffined	4	12.9		14.3		9.2	
	7	15.4		15.0		7.3	
Whole	4	13.3	10.0	14.7	9.2	9.0	3.7
	7	14.6	13.3	12.6	6.7	5.2	3.1
Cut and planted	4	16.6	12.7	18.6	7.3	8.5	2.8
	6	21.2	12.3	22.8	7.7	15.7	4.5
Cut and put in moist chamber	4	16.9		16.7		7.2	
	6	23.5		18.9		15.2	
Cut surface paraffined	4	12.3		12.5		6.3	
	6	15.4		15.8		12.1	
Whole	4	12.3	11.9	12.6	8.9	6.5	5.2
	6	14.6	11.9	13.4	8.1	9.3	2.5

ments was that the cutting admitted additional oxygen to the tissue. In order to test this and to see if oxygen played a part or whether only cutting was necessary, the cut surface was covered with paraffin immediately after cutting and the changes compared with the changes in pieces stored in air or kept in moist chambers without paraffin. Old Green Mountain tubers were used for these experiments. The data in Table V show that the paraffin definitely retarded the development of glutathione and ascorbic acid, and in most cases the treated paraffined pieces showed the same increase as the treated whole tubers from which they were cut. It appears from this experiment that the paraffin adequately replaced the peel so that the paraffined pieces behaved like whole tubers. Table V also presents additional evidence that glutathione increases equally in cut

pieces whether planted or not and shows that the effect observed in the cut pieces is not due to planting.

In connection with the experiments just described, some of the treated whole tubers after being in air for four days were cut into pieces, part of the pieces placed in nitrogen and part in air in a moist chamber. At this point the ascorbic acid value was 14.6. After two days in nitrogen the ascorbic acid value for the treated sample was 13.5, showing no further increase, while the ascorbic acid value for the sample in air increased to 17.7. Glutathione behaved similarly, increasing only in air.

Since the above experiments indicated that lack of oxygen inhibited the development of glutathione and ascorbic acid, additional experiments were made in which the tubers were kept in nitrogen or the air supply limited by placing the tubers or pieces in a small container so that oxygen might soon be used up in the process of respiration. In the first experiments no provision was made to absorb carbon dioxide and consequently there was a tendency for the material in nitrogen to show injury very quickly. This was remedied by placing 100 cc. of 20 per cent KOH in

TABLE VI

EFFECT OF LACK OF OXYGEN ON THE DEVELOPMENT OF GLUTATHIONE AND ASCORBIC ACID
IN POTATO TUBERS FOLLOWING TREATMENT WITH ETHYLENE CHLORHYDRIN

Tubers used	Days after start of treatment	Conditions after treatment	Ascorbic acid		Non-ascorbic acid reducing substances		Glutathione	
			Treated	Check	Treated	Check	Treated	Check
Green Mountain, old	3	Cut; N₂	10.6		5.4		2.6	
		Cut; air limited	12.5		7.5		3.7	
		Cut; air	19.7		19.5		9.0	
		Whole; air	10.2	11.4	9.8	9.4	5.5	5.5
	8	Cut; air limited*	26.0		25.2		15.1	
		Cut; air	21.7		18.3		11.8	
		Whole; air	17.3	11.8	14.7	9.8	8.5	3.8
Bliss Triumph, new	3	Cut; air	29.6	24.2	15.2	8.6	7.5	3.2
		Cut; N₂	29.4	26.9	7.4	9.9	2.0	4.2
Irish Cobbler, new	5	Whole; air	24.6	26.2	28.2	10.6	19.1	6.0
		Whole; N₂	23.0	28.3	13.8	12.5	6.9	6.1
Green Mountain, new	4	Whole; air	21.6	20.4	21.6	10.8	14.1	5.5
		Whole; N₂	21.8	22.2	15.0	11.4	7.8	5.8
Green Mountain, new	5	Whole; air	26.2	24.4	25.8	11.6	15.8	5.9
		Whole; N₂	18.8	25.3	10.0	12.3	3.3	4.5
	8	Whole; air	26.1	24.2	26.7	11.0	17.1	5.7
		Whole; air**	26.2	—	25.8	—	14.0	—
		Whole; N₂*	33.8	23.7	24.6	15.5	12.8	6.8

* Put in air after first sampling.
** Put in nitrogen after first sampling.

the desiccators and the time that the tissue could be left in nitrogen without injury was considerably extended. All the results reported were obtained using KOH in this manner to absorb the carbon dioxide.

Table VI shows that oxygen is necessary for the development of glutathione and ascorbic acid in the tissue. In the first experiment old Green Mountain tubers were treated by the vapor method and some cut into pieces after treatment and placed in nitrogen, in limited air supply in a small desiccator, and in air. Some were left whole in air as a control in addition to the untreated tubers. After three days no increase in ascorbic acid or glutathione had taken place in nitrogen or with a limited air supply, but a large increase in both had taken place in air. After three days all the samples were placed in air, but the samples that had been in nitrogen rotted. The other samples were analyzed after five more days, eight days from the start, and the sample that had been with limited air supply for three days now showed a large increase in ascorbic acid and glutathione, showing that the lack of oxygen only inhibited the development of glutathione and ascorbic acid temporarily and so long as the lack of oxygen was maintained. The next experiment was made with cut tubers of new Bliss Triumph from Florida. No significant change in ascorbic acid was noted during the experiment even in the sample in air, but a large increase in glutathione took place in the sample in air, while no increase and perhaps a decrease in glutathione occurred in the sample kept in nitrogen.

The last three experiments in Table VI were made with small tubers grown in the greenhouse. As is characteristic of whole tubers, especially new tubers with a high ascorbic acid content, no significant increase in ascorbic acid due to treatment with ethylene chlorhydrin was noted during the experimental period even in the sample kept in air. However, these tubers showed a large and prompt increase in glutathione when left in air, but no increase when kept in nitrogen. In the last experiment the tubers were analyzed after five days and then the tubers in nitrogen were placed in air for three more days. A large increase in glutathione then took place in the treated sample. A part of the tubers that had been in air for five days were placed in nitrogen for three more days. No further increase in glutathione took place. The glutathione results of this last experiment are shown in Figure 1. It will be seen that an increase in glutathione occurred only when the tubers were exposed to air.

The effect of lack of oxygen after treatment on the pH of the expressed juice apparently depended on the tubers used. In the case of the southern-grown Bliss Triumph tubers, keeping in nitrogen prevented the usual pH change. However, in the case of small Green Mountain or Irish Cobbler tubers grown in the greenhouse, a pH increase took place in the tubers stored in nitrogen after treatment to the same extent as in air. Nevertheless, in these tubers the glutathione increase was prevented by the lack of

oxygen. This result shows that the action of lack of oxygen in preventing the glutathione increase is not necessarily on the pH increase with which the increase in glutathione appears to be correlated, but probably on some other part of the process of glutathione formation.
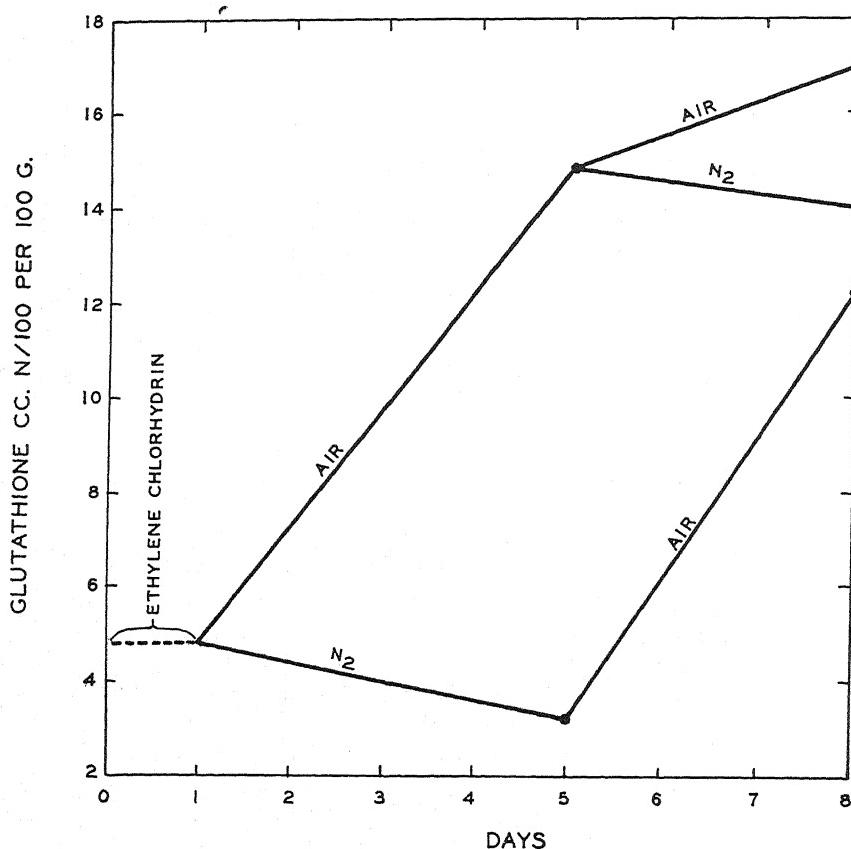
FIGURE 1. Effect of lack of oxygen on the development of glutathione following treatment with ethylene chlorhydrin.

These results showing that oxygen is necessary for the increase in glutathione and ascorbic acid that follows treatment with ethylene chlorhydrin and the marked effect of cutting on the development of these compounds in the tubers naturally led to experiments in which pure oxygen was compared with air. If the reason for the retarding of the increase in ascorbic acid and glutathione in the whole tubers was due merely to a partial impermeability of the whole tubers to oxygen, then increasing the oxygen should act like cutting and hasten the glutathione and ascorbic acid increase. Table VII shows, however, that this was not the case, since

TABLE VII

EFFECT OF INCREASED OXYGEN ON THE DEVELOPMENT OF GLUTATHIONE AND ASCORBIC
ACID IN POTATO TUBERS FOLLOWING TREATMENT WITH ETHYLENE CHLORHYDRIN

Tubers used	Stored after treatment in	Days after start of treatment	Ascorbic acid		Non-ascorbic acid reducing substances		Glutathione	
			Treated	Check	Treated	Check	Treated	Check
Green Mountain, old	Oxygen	4	10.5	11.3	11.1	11.1	6.3	3.9
		7	11.0	10.6	13.0	8.6	7.8	3.7
	Air	4	13.8	12.3	14.2	9.3	9.0	4.3
		7	13.8	11.3	14.2	8.7	8.6	4.1
Bliss Triumph, new, from Cuba	Oxygen	4	24.4	28.3	7.6	8.5	3.2	3.9
		7	21.0	26.8	10.2	8.4	3.5	4.5
	Air	4	29.6	35.0	8.0	9.8	3.2	4.5
		7	29.9	31.0	9.3	13.6	4.9	8.2

keeping the tubers in pure oxygen did not hasten the increase in gluta-
thione or ascorbic acid in old Green Mountain whole tubers or whole
tubers of new Bliss Triumph. If pure oxygen had any effect on such in-
creases it appears to be a slightly adverse one. With these results in mind,
it is necessary to conclude that the effect of cutting is not entirely due to
the admission of oxygen, but that some interaction between oxygen and
the freshly exposed surface is necessary for cutting to be effective in hasten-
ing the increase in glutathione and ascorbic acid.

ASCORBIC ACID AND GLUTATHIONE IN SPROUTING TUBERS

Since the main conclusion of Pett (9) was that ascorbic acid and
glutathione increased in potato tubers during sprouting, and presumably
as a result of sprouting, an attempt was made to repeat the experiments on
which his conclusion was based. He reports that soon after cut pieces
were planted in moist sand at 15° C. an increase in ascorbic acid and
glutathione took place which reached a maximum in about two days. A
decrease to the level of the non-sprouting tubers then took place. The pre-
ceding tables contain several cases where comparison can be made between
untreated, planted pieces and whole tubers of non-dormant material. In
some cases the ascorbic acid content of the cut, planted pieces was slightly
higher than that of the whole tubers, but this increase was only about 10
per cent and much smaller than reported by Pett. In no case was an in-
crease in glutathione observed. This was not due to a difference in ana-
lytical technic, since even if the non-ascorbic acid reducing substances
were considered to be glutathione, no increase was observed. In order to
check this further, three lots of Bliss Triumph tubers and one lot of
Spaulding Rose that had been stored about one month after purchasing
and were just emerging from their rest period were cut up and planted in

moist sand in a greenhouse at about 15° C. A lot of old Green Mountain tubers was also used. At the same time whole tubers of the same lots were placed in sacks in the same greenhouse. Analyses were made after two days and again after three, five, or six days. As usual the cut surface was removed in a thin layer from the cut pieces prior to taking a sample for analysis. The cut surface was not removed by Pett prior to his analyses, and it will be shown that this difference in technic partly explains the results he obtained. The data obtained in the above experiment are given in Table VIII. It will be seen that no large increase in ascorbic acid or glutathione took place in the cut planted pieces.

TABLE VIII

EFFECT OF CUTTING AND PLANTING ON THE ASCORBIC ACID AND GLUTATHIONE
CONTENT OF POTATO TUBERS

Tubers used	Days from start	Ascorbic acid		Non-ascorbic acid reducing substances		Glutathione	
		Cut and planted	Whole, un-planted	Cut and planted	Whole, un-planted	Cut and planted	Whole, un-planted
Bliss Triumph, from Princeton, Fla.	2	18.3	19.1	7.3	8.1	2.8	2.8
	5	15.2	18.1	5.6	9.2	2.2	3.5
Bliss Triumph, from Belle Glade, Fla.	2	17.6	18.8	8.0	9.2	2.5	3.3
	5	16.9	20.9	7.1	7.9	2.5	2.8
Bliss Triumph, from Cuba	2	19.1	17.4	8.1	8.2	2.7	2.7
	5	16.2	24.2	5.4	7.0	2.5	3.0
Spaulding Rose	2	19.3	17.4	7.9	8.2	3.5	3.4
	3	19.0	19.2	6.6	7.2	3.4	4.0
Green Mountain	2	11.0	10.6	7.4	7.9	2.4	2.3
	6	10.8	10.6	6.8	7.6	3.1	—

Since Pett did not remove the cut surface, some experiments were tried in which the cut surface was included in the sample or analyzed separately. Some of these experiments have been presented in Table IV in the section on the effect of cutting. It was noted that ascorbic acid showed a large increase in the cut surface of untreated tubers on the second and third days, after which it fell off rapidly. The ascorbic acid content of the cut pieces was also somewhat higher than that of the whole tubers, especially when the cut surface was included in the sample. In additional experiments, cut planted pieces including the cut surface were analyzed and compared with whole tubers. In order to show more clearly that the increase in ascorbic acid observed was due to a change at the cut surface, thin slices about 3 mm. thick of the same tubers were kept in moist chambers and analyzed at intervals. The results are shown in Table IX. In the case of the old Green Mountain tubers and also of the Bliss Triumph

TABLE IX

EFFECT OF CUTTING ON THE DEVELOPMENT OF ASCORBIC ACID AND OTHER
REDUCING SUBSTANCES IN UNTREATED POTATO TUBERS

Tubers used	Days after cutting	Ascorbic acid			Non-ascorbic acid reducing substances		
		Thin slices	Cut pieces	Whole tubers	Thin slices	Cut pieces	Whole tubers
Green Mountain, old	2	25.2	14.2	12.2	8.4	10.6	9.4
	3	16.7	15.5	12.2	9.7	10.1	10.2
	4	12.6	15.8	11.8	8.2	9.0	9.8
Bliss Triumph, stored 1 month 5° C.	2	18.1	11.9	9.0	4.3	5.7	8.1
	3	12.3	11.9	8.2	5.5	4.9	9.6
	6	6.1	7.7	8.1	9.1	7.5	10.3
Spaulding Rose, new	2	28.5	29.7	31.7	7.5	9.5	12.5
	3	19.9	27.6	26.8	7.3	9.2	9.2
	4	11.0	28.8	29.7	8.2	8.8	7.1

tubers that had been stored at 5° C., an increase in ascorbic acid took place in the cut pieces analyzed including the cut surface. A much larger increase in ascorbic acid took place in the thin slices on the second day, but this increase was only temporary. The non-ascorbic acid reducing substances, which include glutathione, showed no increase in the cut pieces and in some cases a decrease, especially in the thin slices. The new tubers of Spaulding Rose were high in ascorbic acid as is characteristic of new tubers. No increase in ascorbic acid or in non-ascorbic acid reducing substances was observed with these tubers. A rapid decrease in ascorbic acid took place in the thin slices.

The large increase in ascorbic acid that takes place in the thin slices of old tubers in two days might prove to be a convenient method for increasing the antiscorbutic value of potato tubers for dietary purposes late in the season when the ascorbic acid content is low.

The above results show that the increase in ascorbic acid that occurs when potato tubers are cut and planted is not due to sprouting, but to a change that takes place at the cut surface. It is also easy to show that the increase in ascorbic acid and glutathione induced by treatment with ethylene chlorhydrin is not due to the sprouting caused by the treatments, since pieces cut in such a way as to have no eyes show a large increase in both ascorbic acid and glutathione following dip treatment with ethylene chlorhydrin. These results are shown in Table X.

CHANGES IN ASCORBIC ACID ON GLUTATHIONE DURING STORAGE FOLLOWING ETHYLENE CHLORHYDRIN TREATMENTS

In most of the experiments the changes that occurred in the tubers were followed for only a few days after treatment. Consequently, an investigation was made of the changes taking place during longer periods of storage.

TABLE X

EFFECT OF ETHYLENE CHLORHYDRIN TREATMENTS OF PIECES WITHOUT EYES ON THE
GLUTATHIONE AND ASCORBIC ACID CONTENT

Tubers used	Days after start of treatment	Ascorbic acid		Non-ascorbic acid reducing substances		Glutathione	
		Treated	Check	Treated	Check	Treated	Check
Green Mountain, old	5	17.4	14.1	17.8	6.7	8.8	3.4
	8	17.6	12.2	16.8	7.0	7.9	2.1
Irish Cobbler, old	6	19.3	10.0	23.9	8.4	15.1	3.7

It was especially important to do this with whole tubers because the
changes took place more slowly in these. Ascorbic acid usually did not
increase in whole tubers prior to the conclusion of the experiments. How-
ever, in some cases, the last sample taken at about one week after treat-
ment was slightly higher than the check. For these reasons, experiments
were made in which tubers were treated with ethylene chlorhydrin and
then analyzed at longer intervals after treatment than previously. These
results are shown in Table XI. In the first experiment, old Green Moun-

TABLE XI

DEVELOPMENT OF GLUTATHIONE AND ASCORBIC ACID DURING STORAGE OF NON-DORMANT
POTATO TUBERS FOLLOWING TREATMENT WITH ETHYLENE CHLORHYDRIN

Tubers used	Storage conditions	Days from start	Ascorbic acid		Non-ascorbic acid reducing substances		Glutathione	
			Treated	Check	Treated	Check	Treated	Check
Green Mountain, old	Stored at about 25° C.	4	13.3	10.0	14.7	9.2	9.0	3.7
		7	14.6	13.3	12.6	6.7	5.2	3.1
		19	19.2	12.2	16.0	7.7	11.1	4.1
		47	16.9	13.5	11.9	8.9	8.1	4.0
	4 days at about 25° C., then stored at 10° C.	4	13.3	10.0	14.7	9.2	9.0	3.7
		22	20.3	13.8	19.7	8.6	12.5	4.3
		47	24.0	13.0	20.0	8.6	13.9	4.2
		71	20.0	14.5	20.0	9.5	13.1	7.6
		105	14.3	11.7	16.9	10.7	11.3	5.8
Green Mt., old, 1 month later	6 days at about 25° C., then stored at 10° C.	0		13.2		8.8		4.1
		27	18.3	12.6	18.5	9.0	11.2	5.3
		58	15.5	10.6	18.1	11.6	11.8	5.5
Bliss Tr., from Florida, new	6 days at about 25° C., then stored at 10° C.	0		30.2		9.0		3.6
		27	22.2	18.0	15.4	9.2	8.8	4.6
		58	19.9	15.1	16.1	7.3	11.1	4.4

tain tubers with an originally low ascorbic acid content showed a large
increase in ascorbic acid in the treated tubers sampled after 19 days when
stored at 10° or 25° C. Since the tubers kept better at 10° C. and the

experiments could therefore be continued longer, this temperature was used in the other experiments. In the first experiment the treated sample in 10° C. was much higher in ascorbic acid than the check sample even after 71 days and somewhat higher after 105 days. Glutathione also maintained a high level in the treated sample even after 105 days. In the second experiment the same Green Mountain tubers were treated one month after the start of the first experiment, with similar results. In the third experiment new Bliss Triumph tubers from Florida were used. In this case the original ascorbic acid content was high and consequently ascorbic

TABLE XII

CHANGES IN GLUTATHIONE AND ASCORBIC ACID DURING STORAGE OF UNTREATED TUBERS

Tubers used	Days	Ascorbic acid	Non-ascorbic acid reducing substances	Glutathione
Bliss Triumph, from Princeton, Fla.	0	28.6	9.0	4.8
	7	30.2	9.0	3.6
	25	18.6	8.7	3.7
	50	21.7	11.2	5.0
Bliss Triumph, from Belle Glade, Fla.	0	35.3	7.5	3.4
	16	31.4	9.2	4.9
	23	31.3	8.8	4.3
	50	19.8	8.6	3.1
Bliss Triumph, from Cuba	0	33.1	11.7	6.4
	32	20.8	7.6	2.9
Irish Cobbler, 2nd crop, from N. J.	0	19.1	7.1	2.7[*]
	10	17.8	6.8	2.5[*]
	21	15.3	7.2	2.8[*]
	73	10.6	7.9	3.7
Spaulding Rose	0	18.8	7.6	3.4
	7	17.7	7.5	3.8
	20	17.4	8.1	3.4

* Analyses made without adding KCN, hence are probably low.

acid decreased during storage, but less rapidly in the treated sample than in the check sample. In other experiments no glutathione change was obtained with this lot of tubers up to about one week after treatment. This experiment shows that after 27 days a large glutathione increase occurred, which was maintained until 58 days or more after treatment.

These results show that the increase in ascorbic acid and glutathione following treatment with ethylene chlorhydrin is a lasting one and is maintained for one or two months. Since this change takes place in whole tubers, although slowly, it should be possible to increase the ascorbic acid content of old tubers that have a low ascorbic acid content, by vapor treatment with ethylene chlorhydrin. Whether such tubers could be used for food would depend on how much ethylene chlorhydrin remained in

the tubers after the ascorbic acid had increased and on the toxicity of ethylene chlorhydrin.

During the course of the work analyses were made at intervals on the various lots of tubers. Table XII gives the results of these analyses, from which some conclusions concerning the changes in storage can be drawn. Ascorbic acid decreased during storage as has been observed by others (2,8,9). There was, with one possible exception, no evidence of any large change in the glutathione content or in the amount of non-ascorbic acid reducing substances. However, the tubers used had been harvested for an uncertain length of time before they were purchased, so the results have no bearing on any change in glutathione that may have taken place just after harvest. The results on old tubers, which had been stored for a long period prior to purchase, are not included in the table since they showed no significant change in either ascorbic acid or glutathione after further storage.

DISCUSSION

The results presented above confirm the findings of Pett (9) which indicated that treatment with ethylene chlorhydrin increases the ascorbic acid content of potato tubers and are in agreement with previous experiments (5) which showed that glutathione increased in potato tubers following treatment with ethylene chlorhydrin. A number of factors, such as cutting, oxygen, age of the tubers, and storage have been investigated. The results do not conflict factually to any large extent with those presented by Pett except insofar as no large increase in ascorbic acid or glutathione was noted due to cutting and planting untreated tubers so that sprouting might take place. Since, however, in a few cases a small increase in ascorbic acid was observed in cut planted pieces, especially if the cut surface was included in the analyses, the results of Pett on this phase of the problem may have been due to using tubers that responded in an unusual manner.

The additional findings presented make untenable Pett's conclusion that glutathione and ascorbic acid increase in potato tubers because of sprouting. The increase in glutathione and ascorbic acid following treatment with ethylene chlorhydrin is clearly not caused by the process of sprouting, since it takes place equally well in pieces of tubers cut in such a way that no eyes are present. Furthermore, the change in glutathione and ascorbic acid in cut pieces of the treated sample begins at the cut surface, showing that cutting plays an important part in these changes. In cut pieces of non-dormant, untreated tubers, an increase in ascorbic acid occurs in the cut surface on about the second day after cutting. It is likely that the increase in ascorbic acid that Pett found about the same time after planting was due to the effect of cutting and not to the sprouting of the tubers.

The results make available two methods for increasing the ascorbic acid content of old potato tubers. One is to treat with ethylene chlorhydrin and then store for about three weeks, and the other is to cut the tubers into slices and store in moist air for two days. From a dietary standpoint the value of the first method would depend on whether any ethylene chlorhydrin or other toxic substance remained in the tubers after the ascorbic acid increase had taken place and the value of the second would be limited by the natural objection to eating cut slices, the surface of which would be darkened. It is also well to point out that the ascorbic acid results depend on the specificity of the 2,6-dichlorophenolindophenol titration and it is not beyond the bounds of probability that the changes observed may be due to some substance other than ascorbic acid. Biological tests would give an answer to this.

The necessity of oxygen for the formation of glutathione in potato tissue is another bit of evidence for the close connection between this compound and respiration, but not as a controlling factor, since previous results show that the increase in respiration occurs before the glutathione increases (7). However, the high glutathione content following ethylene chlorhydrin may in some way be a result of the increased respiration. The correlation between the increase in glutathione and the increase in pH reported previously (5) and strengthened by the results with butyl bromide presented in the present paper indicate that the steps that lead to the glutathione increase may be: first, an increase in respiration; second, a decrease in citric acid due to its acting as a substrate in the respiratory process; third, an increase in pH due to loss of citric acid; and fourth, an increase in glutathione brought about in some way by the greater alkalinity of the tissue.

SUMMARY

1. A modification of the sulphur reduction method for the determination of glutathione gives lower results on the same metaphosphoric acid extracts of potato tissue than the iodometric method, even when this is corrected for ascorbic acid. This is interpreted to mean that iodine-reducing substances other than glutathione and ascorbic acid are present in potato tissue.

2. Ethylene chlorhydrin treatments do not increase the ascorbic acid content of freshly-harvested potato tubers, which have a high ascorbic acid content. However, if the tubers are cut into pieces after treatment, the ascorbic acid content is maintained at its originally high level in the treated pieces, but a large decrease takes place in the untreated pieces.

3. Ethylene chlorhydrin treatments slowly increase the ascorbic acid content of old tubers which have a low ascorbic acid content, about 20 days at 10° C. being required for the increase to reach its maximum, after

which ascorbic acid is maintained at a higher level than the untreated tubers for over a month. However, if the tubers are cut into pieces after treatment a large increase in ascorbic acid takes place in the treated sample within three days.

4. The increase in glutathione that follows treatment of potato tubers with ethylene chlorhydrin is hastened by cutting the tubers into pieces. To be effective cutting must follow or immediately precede the treatment.

5. Butyl bromide treatments produce a large increase in glutathione if the tubers are cut into pieces. This result is predictable from the previously observed increase in pH with which the glutathione increase is correlated.

6. The increase in glutathione and ascorbic acid that occurs in pieces cut from tubers treated with ethylene chlorhydrin begins at the cut surface. A large increase in ascorbic acid also takes place after two days in the cut surface of untreated old tubers, but is maintained for only a few days.

7. The ascorbic acid content of the tissue of old tubers may be approximately doubled within two days by cutting into thin slices and storing in moist air.

8. Covering the cut surface with paraffin or placing in nitrogen retards or prevents the increase in glutathione or ascorbic acid. Keeping in nitrogen also prevents the increase of glutathione in whole tubers. However, the increase in ascorbic acid and glutathione takes place upon returning the tissue to air.

9. Although oxygen is necessary for the development of ascorbic acid and glutathione in potato tubers, the effect of cutting is not due entirely to the admission of extra oxygen to the tissue, but rather to some interaction between oxygen and the freshly exposed cut surface, since placing whole tubers in pure oxygen following treatment with ethylene chlorhydrin did not hasten the rate of increase of glutathione or ascorbic acid above that observed in air.

10. The increase in glutathione and ascorbic acid in cut planted pieces following treatment with ethylene chlorhydrin is not due to the sprouting that follows these treatments, since these changes take place in pieces cut without eyes.

11. The small increase in ascorbic acid that follows cutting and planting of non-dormant tubers is due to the cutting and is not a result of sprouting, since this increase is localized at the cut surface. No increase in glutathione takes place following cutting and planting.

12. As reported by others, ascorbic acid decreases in potato tubers during storage. However, a parallel decrease in glutathione usually does not occur.

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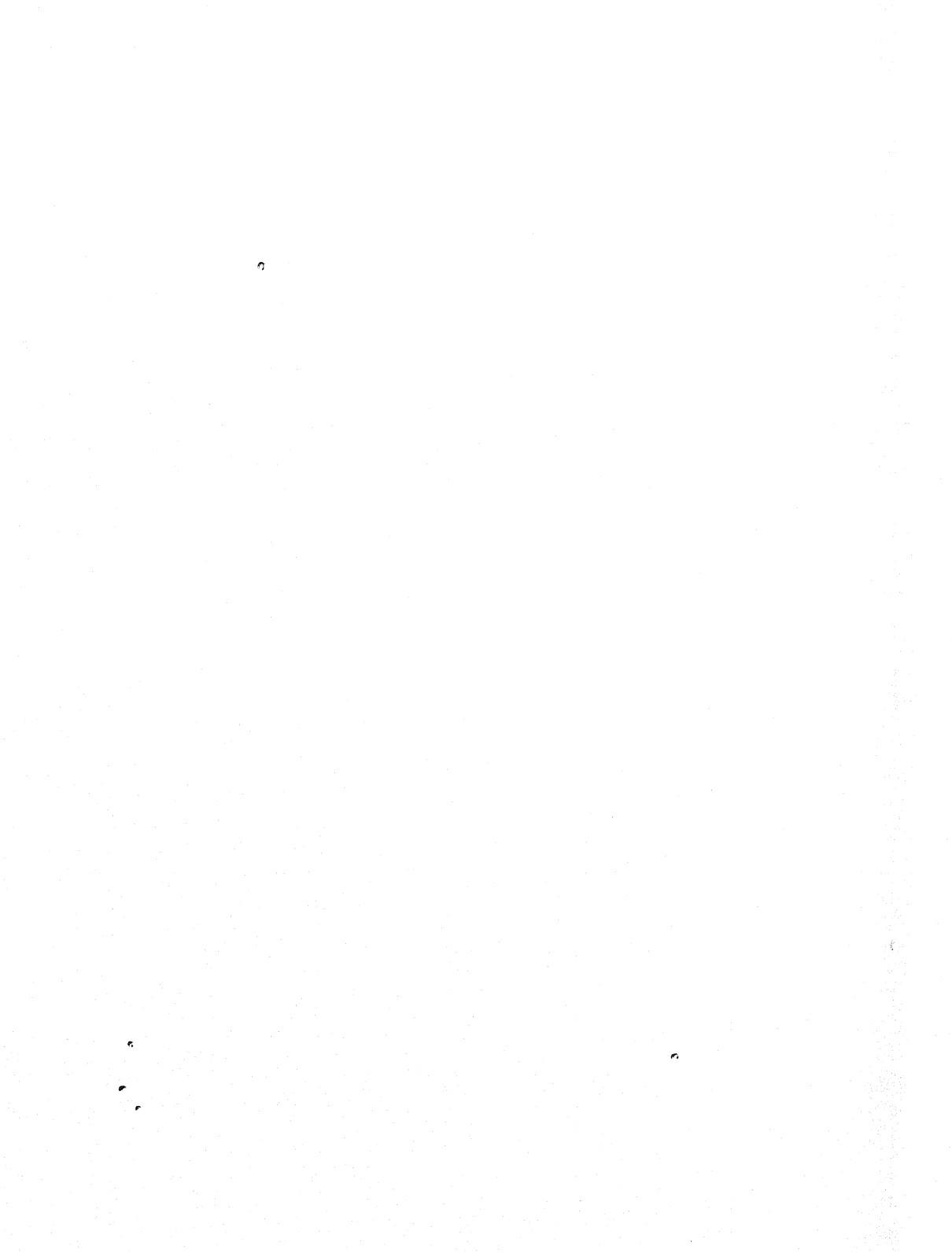
USE OF INCOMPLETE BLOCK REPLICATIONS IN ESTIMATING TOBACCO-MOSAIC VIRUS

W. J. YOUDEN

INTRODUCTION

Wherever experiments are performed a great deal of attention is directed to the selection of uniform starting material, the fair apportionment of it into lots, and the maintenance of equality of opportunity thereafter among the several lots save in respect to the factors under test. This leads, in the case of field trials, to the use of compact blocks and severe restrictions as to the number of items simultaneously compared lest the blocks be so large as to introduce a considerable element of soil heterogeneity. Customarily all the items are included in the block and the block replicated as many times as seems desirable. Afterwards the block characteristics are eliminated from the estimate of error by the use of Student's method or the analysis of variance. The limitation to a small number of the items to be compared is an irksome condition in much experimental work.

The methods employed for the estimation of the relative infective capacity of a series of virus preparations encounter the same difficulties. The test leaves from different plants and even from the same plant show great variations in the production of lesions. The two halves of the same leaf are more nearly alike so these are often used to compare two virus preparations. If it is desired to evaluate more than two preparations simultaneously one may be selected as a standard and applied to one-half of every leaf and the other halves apportioned among the rest of the solutions. It is clear that the difference between any solution and the standard is obtained by taking the difference between two half leaves from the same leaf, but that a comparison of two solutions, neither of which is the standard, involves four half leaves. Such comparisons are subject to twice the error of the simple differences. Alternatively the solutions could be paired in every possible way and the various pairs assigned to whole leaves. The leaves then constitute incomplete blocks because any one block does not contain all the solutions tested. Using this arrangement the whole series of solutions may be put on an equal footing and the leaf characteristics eliminated from the results.

The method of incomplete blocks is described in recent papers by Yates (2, 3), and referred to by Fisher (1, p. 141). This paper presents a modified form of arranging the blocks and an example of its application to the estimation of the infectivity of virus preparations.

METHOD OF INCOMPLETE BLOCKS

When the incomplete block is made up of three or more items the apportionment of the items under test among the several blocks is such that every possible pair is formed as before. For example, with seven treatments arranged in blocks of three, two of the possible arrangements are:

```
A  B  C  D  E  F  G          A  B  C  D  E  F  G
B  D  F  E  G  A  C          B  C  D  E  F  G  A
C  F  E  A  B  G  D          D  E  F  G  A  B  C
```

The incomplete blocks consist of the vertical rows of three letters, and since there are seven blocks there are three replications of each treatment. The modification of the arrangement here introduced is the arrangement of the seven blocks side by side so that the seven treatments also appear in the form of three complete replications in the horizontal rows. That is, from this point of view there are three replicated blocks each containing all the treatments. In the analysis of variance these complete block effects may also be eliminated and a further reduction in the error term obtained. The seven columns may be arranged in any order and the three rows may be interchanged to give additional arrangements.

The method of eliminating block effects may be made clear by an example, in which seven blocks each containing three plots

1	2	3	4	5	6	7
$A=18$	$B=76$	$C=62$	$D=66$	$E=47$	$F=112$	$G=55$
$B=77$	$D=86$	$F=93$	$E=95$	$G=86$	$A=95$	$C=80$
$C=30$	$F=83$	$E=115$	$A=112$	$B=65$	$G=70$	$D=103$

or treatments gave the values shown. Blocks 1, 2, and 5 respectively, are used to evaluate the following expressions:

$$2B-(A+C)=154-(18+30)=106$$
$$2B-(D+F)=152-(86+83)=-17$$
$$2B-(E+G)=130-(47+86)=-3$$

These differences are the result of comparisons within a block and may be likened to the practice often followed with Student's method of taking the difference between the individuals constituting a pair. The sum of these three expressions is

$$6B-(A+C+D+E+F+G)=86.$$

This may be written in the alternative form

$$7B-(A+B+C+D+E+F+G)=86.$$

Upon dividing by 7 there is obtained

$$B-\text{mean of all}=12.3$$

$$\text{or } B=\frac{1626}{21}+12.3=89.7.$$

Here B stands for the mean value of treatment B, and 1626 is the total of all the data. This procedure may be followed for the other six treatments and the mean values of the treatments before adjustment for block effects compared with the values after adjustment.

Treatment	A	B	C	D	E	F	G	Mean
Before adjustment	75.0	72.7	57.3	85.0	85.7	96.0	70.3	77.4
After adjustment	75.0	89.7	60.7	78.7	81.6	87.6	66.0	77.4

Since the experiment was actually a uniformity trial, the treatments being identical, the same value should be expected from all treatments and this is more nearly the case after eliminating the block effects.

The results of the arithmetical process just described may be obtained in a more convenient manner as will be shown on another set of data. These data were obtained in the course of an investigation of the infective power of crystalline preparations of tobacco-mosaic virus. The infective capacity of a solution of these crystals is estimated by counting the spots or local lesions (Fig. 1) which appear on the leaves of *Nicotiana glutinosa* L. plants when they have been rubbed with a cloth moistened with the solution. Previous work (4) had shown that the counts obtained in this way were extremely variable. Not only did the test plants vary greatly but leaves from the same plant showed a gradient which was consistent throughout a given lot of plants. In the early work a Latin Square arrangement of the solutions under test was found advantageous. Individual plants became columns in the Latin Square and the leaf positions corresponded to the rows. The size of the square, and consequently the number of solutions tested simultaneously, was limited since only five or six suitable leaves are found on a plant. The modified form of incomplete blocks was found admirably adapted to this work. Block, or plant effects, and the influence of leaf position could both be avoided while the number of test preparations that could be simultaneously compared was increased to 21 using plants with five leaves.

In this experiment the 21 test preparations were made up of seven different concentrations of the virus crystals in water, 0.05 molar and 0.10 molar neutral phosphate buffer. The virus concentrations formed a geometric series, each solution being one-half the concentration of the preceding one. The counts made on the 105 leaves from 21 plants are shown in Table I. The leaves from five of the plants are shown in Figure 1 where they are arranged in order of their position on the plant. The letters refer to the code designation of the solutions, the figures to the number of lesions counted. The five plants selected are those with the five replicate leaves which received solution P. Examination of Figure 1 will show that solution P was applied at each of the five leaf positions and that all the remaining 20 test solutions are accounted for on the rest of the leaves.

The data in Table I, while not in a compact form, are arranged to facilitate the computations. The particular sets of 21 counts which came from each leaf position are not apparent but are easily obtained from the original notebook record of the counts. The spaces in the table which have entries show which solutions were applied to each plant. The next to last column requires explanation. Each entry is the sum of the five plant totals of the plants which received the solution designated in the first column. The first
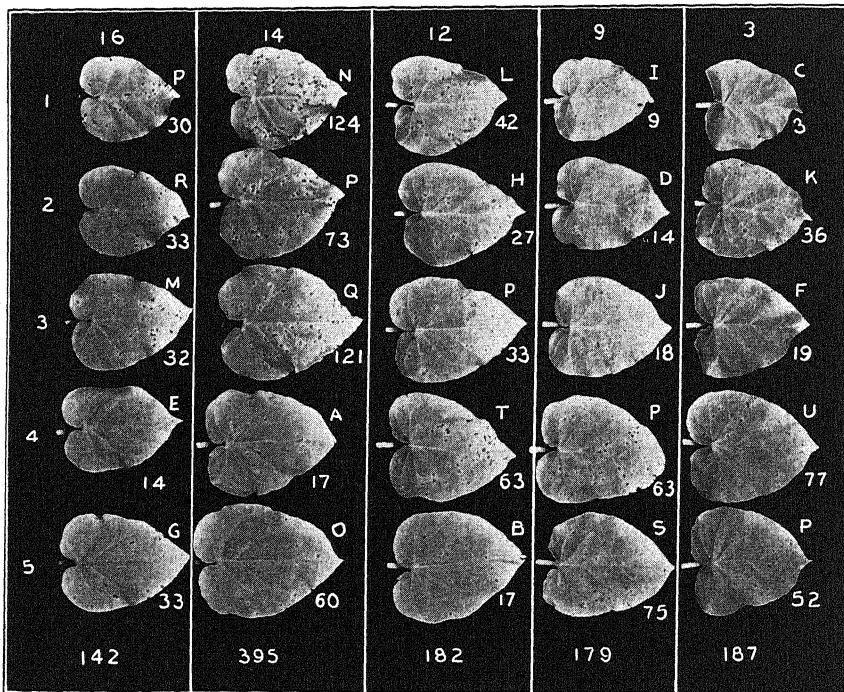
FIGURE 1. *N. glutinosa* leaves with local lesions from five of the plants used in determining the infectivity of virus preparations by the method of incomplete blocks. The leaves from each plant constitute an incomplete block.

entry, 1293, is the sum of the totals for plants 1, 6, 11, 14, 19. The entries in the last column are obtained by subtracting the totals for five plants from five times the solution totals. For example, $(5 \times 82) - 1293 = -883$. The differences so obtained are the correction terms which must be divided by 21 and added or subtracted to the general mean to give the adjusted values. The mean value for A is $4932/105 - 883/21 = 4.9$. The mean values are listed in Table II together with the distinguishing code letters. Below Table I are listed the sum of the squares of the counts for the 105 leaves, the 21 plant totals, the five leaf position totals, and the 21 correction terms. The five leaf position totals were 702, 916, 1150, 1083,

TABLE I

LESION COUNTS FROM N. GLUTINOSA PLANTS INOCULATED WITH TOBACCO-MOSAIC VIRUS

Sol'n.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Sol'n total	Total 5 plants	Corr.
A	5 25 32 43 28					13	21				41	17	16	17					6			82	1293	—883
B		7	3	14				39		20		17			35	14	21	27		17	32	86	882	—452
C			19	35	13 14	20 26	20	63	14	28		27	18		33	33	37	27		20	32	130	1355	—705
D						35 30			9 18	26	60 62 100		18					75				130	1007	—357
E			36	27	40		78	63 132			55	42	21	124 60 73 121	75	32	42			54	55	102	927	—417
F	32			29	45				63	85		33	54		24	30	24	70 44	100 64 71 82	70	81	120	846	—246
G		45	52	117	33		70	191	75	120		63	46		133	33	123			57	30	148	987	—247
H		39					55															180	1326	—426
I																						110	976	—426
J																						191	1201	—246
K			77																			305	1210	315
L																						312	1175	385
M																						198	1325	—335
N																						446	1499	731
O																						268	1450	—110
P																						251	1085	170
Q																						314	1185	385
R																						355	1170	599
S																						418	1379	711
T																						431	1192	963
U																						355	1184	591
Total	133	168	187	222	145	124	244	488	179	289	318	182	155	395	300	142	247	243	323	218	230	4932	24060	0

	Leaf Counts	Plant Totals	Position Totals	Corrections	Grand Total
Sum of squares	347936	1329082	4995810	5551582	24324624
Divided by	1	5	21	105	105
Quotient	347936.1	265816.4	237895.7	52872.2	231663.1
Subtract	231663.1	231663.1	231663.1		
	116272.9	34153.3	6232.6		

TABLE II
ADJUSTED VALUES OF LESION COUNTS IN TABLE I

Solution in	Concentration of virus						
	I*	1/2	1/4	1/8	1/16	1/32	1/64
Water	S80.8	P55.1	M31.0	J35.3	G35.2	D30.0	A 4.9
0.05 M PO₄	T92.8	Q65.3	N81.8	K62.0	H26.7	E27.1	B25.5
0.10 M PO₄	U75.1	R75.5	O41.7	L65.3	I26.7	F35.3	C13.4

* This solution contained 1.2 mg. crystals per liter.

and 1081. There are also shown the arithmetical operations needed to obtain the sums of squares for the analysis of variance given in Table III. The mean squares show that considerable differences in infective power

TABLE III
ANALYSIS OF VARIANCE OF DATA IN TABLE I

Item	Degrees freedom	Sum of squares	Mean square	Standard deviation
Plants	20	34153.3	1707.7	
Leaf position	4	6232.6	1558.2	
Solutions	20	52872.2	2643.6	
Error	60	23014.8	353.6	18.8
Totals	104	116272.9		

existed among the solutions tested, and that the contribution of the individual test plants and leaf positions to the variation in the observed counts was considerable. The standard deviation of a single leaf is close to 40 per cent of the average leaf count and is some indication of the difficulties attendant determinations of infectivity. Where greater precision was required the five solutions associated with each plant in this design were applied to five plants in the form of a Latin Square. The treatment totals obtained from the 21 Latin Squares thus formed then constituted the entries in a form exactly like that of Table I. The analysis corrects for the variation found between the Latin Square blocks just as in the present procedure the results were adjusted for plant effects. Ordinarily it would be difficult to obtain 105 plants that were reasonably uniform. It is much less trouble to obtain 21 sets of five plants, the plants within any set being matched. Indeed, even the latter requirement need not be met in this case, because the arrangement of the solutions as a Latin Square within each set disposed of both leaf position and plant effects for the five treatment totals of the set.

DISCUSSION

The modified incomplete block design bears some resemblance to the familiar Latin Square. The disposition of the treatments over the test

area, or in this case plants, is in the form of a rectangle with complete block replications arranged along the long axis and incomplete blocks running across the rectangle. The more important feature is the incomplete block but in certain instances a further gain may be made by eliminating the contribution of the whole blocks. This was the case with the *N. glutinosa* plants where each leaf position carries a complete replication of the solutions under test. Another possible application may be found in diet tests with animals. Here the number of diets which it is desired to compare may exceed the litter size. In this case the diets may be distributed using the litters as incomplete blocks and the results adjusted for the differences between litters. If the animals composing each litter are arranged in order of initial weight, and one complete replication of the diets assigned to the heaviest animal from each litter, another complete replication to the next heaviest animal, and so on, the contribution to the variance arising from the use of animals of different weights may then be removed from the error term.

The application of the incomplete block method to specific problems suffers from the restriction that only certain combinations of block size and total number of treatments are possible. Thus with blocks of four treatments, 13 items may be tested, and with blocks of six, 31 items. If the number of items is just short of the requisite number certain ones may be repeated. Since some patience is required to obtain these arrangements a few examples are listed below.

```
A B C D E F G H I J K L M        A B C D E F G H I J K L M
C D E F G H I J K L M A B        B E J M F K C I L G A D H
D E F G H I J K L M A B C        C H E I A D K J B M L G F
H I J K L M A B C D E F G        D K L E G J I A F B M H C
```

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A B C D E F G H I J K L M N O P Q R S T U
D E F G H I J K L M N O P Q R S T U A B C
E F G H I J K L M N O P Q R S T U A B C D
J K L M N O P Q R S T U A B C D E F G H I
L M N O P Q R S T U A B C D E F G H I J K
```

```
A B C D E F G H I J K L M N O P Q R S T U V W X Y Z a b c d e
F G H I J K L M N O P Q R S T U V W X Y Z a b c d e A B C D E
G H I J K L M N O P Q R S T U V W X Y Z a b c d e A B C D E F G H
I J K L M N O P Q R S T U V W X Y Z a b c d e A B C D E F G H
P Q R S T U V W X Y Z a b c d e A B C D E F G H I J K L M N O
T U V W X Y Z a b c d e A B C D E F G H I J K L M N O P Q R S
```

SUMMARY

A modification of the incomplete blocks described by Yates has been devised, the configuration of which permits the construction of complete blocks of replicates without sacrificing the advantage of the incomplete blocks. The design has been used in studies of the infectivity of solutions of crystalline tobacco-mosaic virus on *N. glutinosa* plants. The requisite computations for the application of the analysis of variance to the data are given.

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DILUTION CURVE OF TOBACCO-MOSAIC VIRUS

W. J. YOUDEN

INTRODUCTION

In an earlier report by Youden, Beale, and Guthrie (7) tobacco-mosaic virus dilution curves from a number of sources were assembled and an attempt made to fit these data to a simple dilution law. It was recognized at the time that the agreement between the observed counts and those calculated from the equation suggested was not entirely satisfactory especially at high dilutions. The investigation did succeed in establishing the concordance of the various reports as to the general form of the dilution curve. Most of these data were obtained by experimental arrangements that were not capable of giving comparable estimates of the relative infectivity of a series of dilutions with the accuracy desired. This is evident from the large intervals employed between points on the curves. Many of the dilutions were made using a geometrical series in which the dilution was ten-fold between adjacent points on the curve. This was necessary in order to show significant changes in the infectivity as a result of dilution.

Recent advances in the design of experiments have made available a variety of devices which are adapted to the very considerable heterogeneity of the test plants and which facilitate the simultaneous comparison of a large number of test solutions. One of these designs was found especially useful and is given in detail in a recent publication (6). The first attempts to obtain more accurate data gave dilution curves which showed marked departures from those expected. The dilution curve was, therefore, investigated more intensively using an arithmetical series of dilutions. The differences in infectivity between adjacent dilutions were now considerably smaller and the work afforded an excellent trial of the new experimental arrangements. The results assure that these methods will prove of value for estimating the infectivity of virus preparations.

MATERIALS AND TECHNIQUE

Needle-shaped crystals (Fig. 1) of tobacco-mosaic virus protein were obtained from tobacco (*Nicotiana tabacum* L. var. Turkish) plants which had been infected with tobacco virus. The procedure outlined by Stanley (2, 3) was followed, omitting the treatment with lead subacetate (4). Some of the work was done with crystals isolated by Dr. Stanley. The crystals were kept in contact with the mother liquor in the cold and at intervals stock solutions were prepared by filtering off the crystals and taking up in water. These solutions were dialyzed in cellophane tubing using a rocking arrangement. The dialysis was continued until tests for sulphate ion and

ammonia using Nessler's reagent were negative. The nitrogen was then determined by a micro-Kjeldahl method and the concentration of protein taken as equal to five times the nitrogen.

Two methods of diluting the stock solutions were used. At first equal quantities of the stock solution were added from a pipette to flasks containing such volumes of distilled water as would give the desired series. The practice was later adopted of varying the ratio of virus stock and water maintaining the final volume constant. This was easily accomplished using two Mohr pipettes, one for the virus stock and one for the water. The solu-
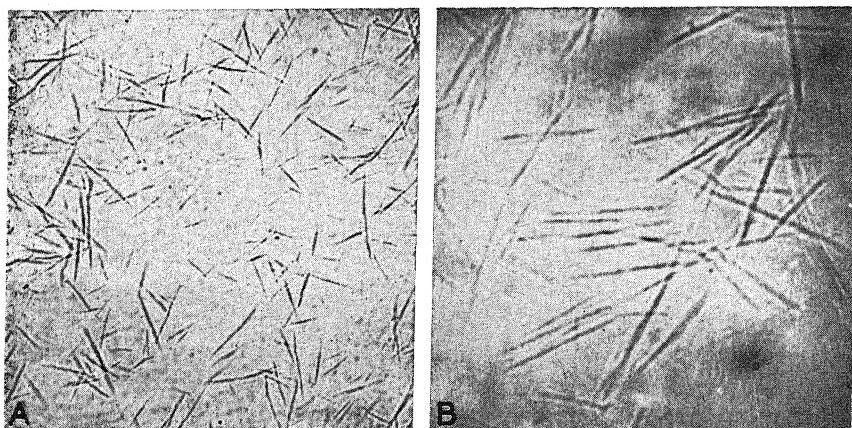
FIGURE 1. Crystals obtained from Turkish tobacco plants infected with tobacco virus. A, ×220; B, ×552.

tions were usually used at once to inoculate *Nicotiana glutinosa* L. plants which had five leaves available after decapitation. The plants were inoculated by wetting a square of cloth, four by five inches folded into four thicknesses, with the solution and gently rubbing the surface of the test leaves with the wet cloth. The leaves were immediately rinsed and the lesions counted after an interval of three to five days.

The number of solutions of which the relative infectivity was to be determined usually exceeded the number of leaves on a plant. Several methods of distributing the test solutions among the leaves were tried. These are listed below as A, B, C, and D, and will be referred to by these letters for convenience.

Method A—Modified incomplete blocks using a single plant as an incomplete block.

Method B—Modified incomplete blocks using Latin Squares as incomplete blocks.

Method C—Incomplete blocks using a leaf as an incomplete block.

Method D—Latin Square.

The first two methods are described fully in a separate paper (6). The use of a leaf as an incomplete block (method C) requires the forming of all possible pairs from the solutions to be compared. The number of pairs which can be made from ten solutions is 45. These 45 pairs may be divided into nine sets of five pairs so that any set of five pairs represents all ten solutions. These form natural sets to allocate to nine plants. One such arrangement is listed below.

AB	BD	GI	CG	IE	JC	DE	AJ	HF
CD	IF	JH	EA	HC	EF	JB	IB	DG
EG	HE	CF	JI	DJ	GA	AH	FD	BC
FJ	GJ	BE	DH	AF	BH	CI	GH	IA
HI	AC	DA	FB	BG	ID	FG	EC	EJ

The two letters in each pair refer to the two solutions applied to the opposite halves of the same leaf. All ten solutions are found on each plant. This arrangement was found highly efficient.

RESULTS OF EXPERIMENTS WITH DILUTION SERIES

The total lesion count for each dilution for 19 different series is given in Table I. The first column gives the number of the experiment and is used to identify the curves shown in Figures 2 and 3. The letters in the second column indicate the method of arrangement of the solutions on the test leaves. The next column shows whether the medium for the virus solution was water or 0.1 molar neutral phosphate buffer. The body of the table gives the concentration of the various dilutions prepared and the corresponding lesion counts obtained. In the last column there is given the standard deviation of the lesion counts. The 19 series, two of which are not given in the table, were obtained over an interval of six months and are listed in chronological order.

Series 1 shows a smooth falling off in infectivity upon dilution until the most dilute solution is reached. This solution, although but half the concentration of the preceding solution, produced nearly as many lesions. The same solutions and more dilute ones prepared from them were used for Series 3 and this series again shows an unexpected small difference between the 0.12 to 0.06 dilutions. The counts for the phosphate preparations (Series 2) appear somewhat erratic. The variable effect of phosphate buffers on the infectivity is mentioned by Stanley (1, p. 914). Series 4 and 5 constituted one experiment with 21 solutions using 105 plants. The dilution steps were made smaller to obtain points in between the 0.06 and 0.12 concentrations. The same equivalence in infectivity of the 0.06 and 0.12 solutions was found with even higher counts for the intermediate dilutions. The crystals isolated by the author were used in this series while the other series were made up with the protein crystals obtained from

TABLE I
DATA FOR DILUTION CURVES OF TOBACCO-MOSAIC VIRUS

Concentration in mg. per cc. and lesion count

No.	Design	Media	Data (concentration : lesion count)	S.D.
1*	A	H_2O	.42 : 2126; .36 : 2141; .30 : 2078; .24 : 1840; .18 : 1541; .12 : 1244; .06 : 1201	179
2*	A	PO_4	.42 : 2495; .36 : 2050; .30 : 1713; .24 : 2287; .18 : 1906; .12 : 1782; .06 : 1143	152
3*	A	H_2O	.24 : 985; .18 : 963; .12 : 704; .06 : 631; .04 : 464; .02 : 438; .01 : 350	57
4	B	H_2O	.40 : 3147; .32 : 2207; .24 : 2265; .16 : 1895; .12 : 1572; .10 : 1895; .08 : 1647; .06 : 1467; .04 : 1105; .02 : 870; .01 : 1541	157
5	B	PO_4	.40 : 3125; .32 : 2808; .24 : 2727; .16 : 2624; .12 : 2281; .10 : 2004; .08 : 2093; .06 : 1675; .04 : 1834; .02 : 1247; .01 : 1032	
6	B	H_2O	.40 : {2671; .32 : 3091; .24 : 2596; .20 : 2481; .16 : 2302 / 2483; .14 : 2446 / 2669; .12 : 1848 / 2191; .10 : 1839 / 2037; .08 : 1614 / 1793; .06 : 1649 / 1667; .04 : 1233 / 1536; .02 : 980; .01 : 497; .005 : 513}	168
8	C	H_2O	.20 : 443; .18 : 587; .16 : 541; .14 : 429; .12 : 414; .10 : 375; .08 : 401; .06 : 371; .04 : 412; .02 : 297	47
9	C	H_2O	.20 : 421; .18 : 511; .16 : 392; .14 : 368; .12 : 344; .10 : 316; .08 : 279; .06 : 318; .04 : 286; .02 : 145	30
10	A	H_2O	.16 : 1578; .14 : 1557; .12 : 1668; .10 : 1313; .08 : 1652; .06 : 1030; .04 : 1242	71
11	A	H_2O	.16 : 1356; .14 : 1378; .12 : 1547; .10 : 1314; .08 : 1681; .06 : 1146; .04 : 1115	83
12	C	H_2O	.40 : 229; .36 : 235; .32 : 295; .28 : 251; .24 : 198; .20 : 195; .16 : 189; .12 : 217; .08 : 146; .04 : 144	24
13	C	H_2O	.40 : 181; .36 : 229; .32 : 244; .28 : 233; .24 : 201; .20 : 171; .16 : 173; .12 : 166; .08 : 131; .04 : 100	27
14	C	H_2O	.15 : 174; .14 : 150; .13 : 236; .12 : 179; .11 : 184; .10 : 208; .09 : 138; .08 : 163; .07 : 190; .06 : 169	21
16*	C	H_2O	1.50 : 305; 1.00 : 271; .67 : 294; .44 : 258; .30 : 253; .20 : 169; .13 : 100; .09 : 117; .06 : 110; .04 : 143	19
17*	C	H_2O	1.50 : 389; 1.00 : 344; .67 : 324; .44 : 250; .30 : 205; .20 : 200; .13 : 149; .09 : 157; .06 : 175; .04 : 228	24
18*	C	H_2O	.25 : 205; .225 : 246; .20 : 204; .175 : 171; .15 : 129; .125 : 123; .10 : 162; .075 : 155; .05 : 128; .025 : 78	17
19*	C	H_2O	.25 : 208; .225 : 307; .20 : 234; .175 : 192; .15 : 188; .125 : 151; .10 : 192; .075 : 155; .05 : 147; .025 : 108	21

* Protein crystals from Dr. Stanley were used for these preparations
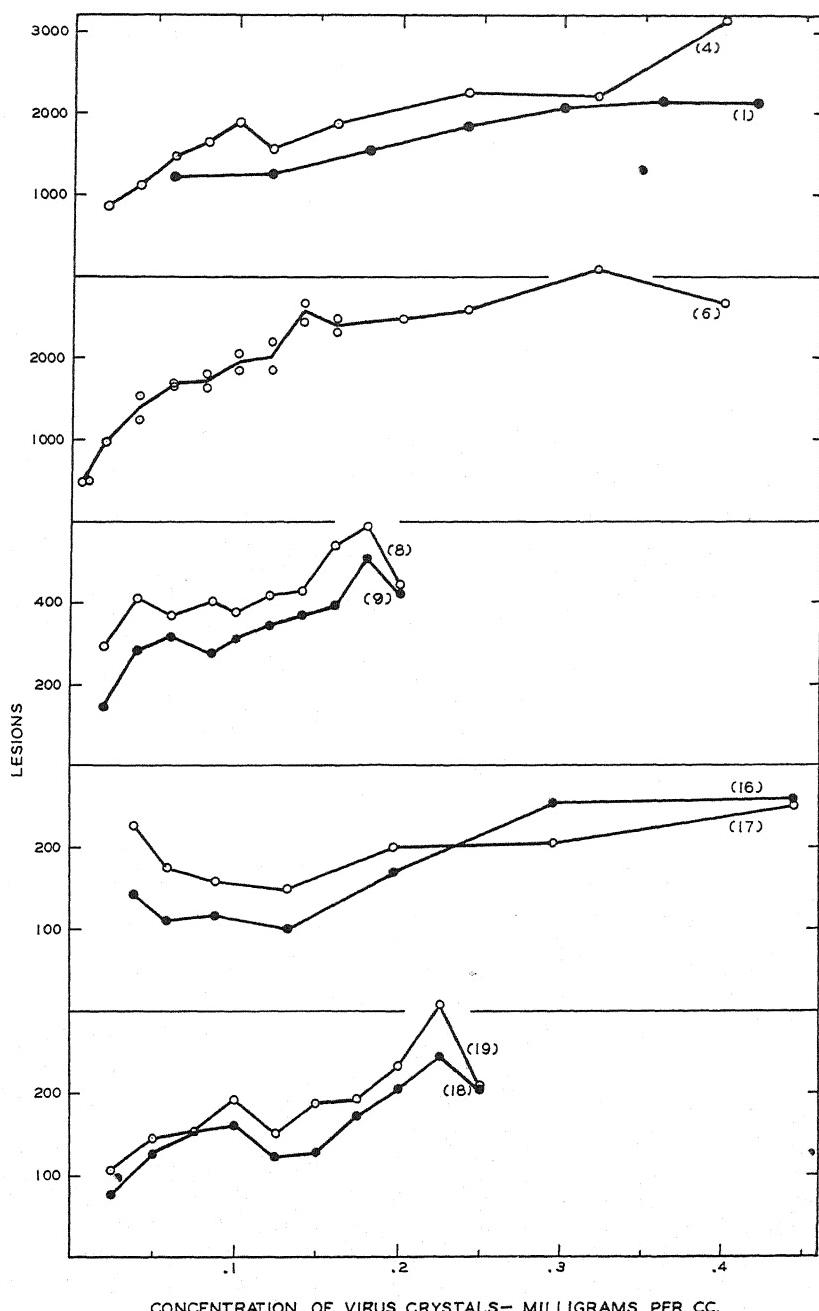
FIGURE 2. Dilution curves showing the change in infectivity of the virus of tobacco mosaic with dilution. Numbers in parentheses correspond to those in Table I.

Dr. Stanley. Series 6 was made up of 14 different dilutions. Seven of these were duplicated making available 50 leaves for the duplicated values as against 25 leaves for the other dilutions. There is, for this series, a less pronounced but still definite indication of a range of unchanged infectivity with dilution. At this stage in the work a series of five solutions, somewhat more dilute than previously used, were arranged in a Latin Square and the square replicated five times. The concentrations of the five solutions were 0.01, 0.008, 0.006, 0.004, and 0.002 and the total counts found were 959, 706, 438, 415, and 190, respectively. The values obtained
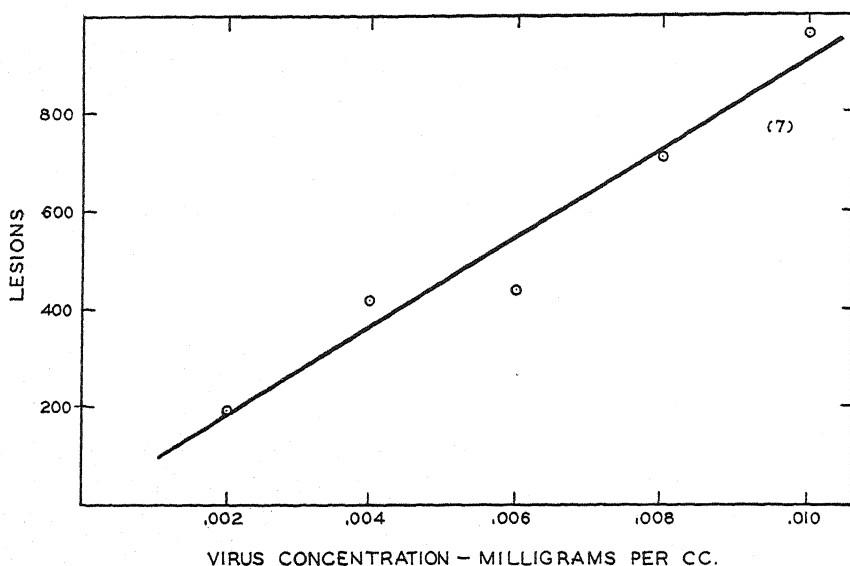
FIGURE 3. Graph showing the linear relation of lesion production to concentration at high dilution. The data are given as Series 7 in the text.

are plotted as Series 7 in Figure 3 and follow a straight line trend within the error of the experiment. This was satisfactory evidence that at dilutions not much below those showing the anomalous behavior the technique was adequate to evaluate small differences. Series 8 and 9 were run on two sets of plants. The same solutions were used for each set and the two sets inoculated at the same time. Only nine plants constitute a set, or less than a plant for each dilution. The curves parallel each other closely and since the two sets of data have nothing in common except the solutions, they lead to the conclusion that the counts actually represent the solutions and not the plants or rubbing technique. Again there appears a level region centering around a concentration of 0.1 mg. per cc. Series 10, 11 and 12, 13 are not plotted but the two numbers of each series follow a common trend

and there is a considerable interval of undiminished infectivity upon dilution. Dilutions by very small steps are shown in Series 14. These counts are scattered around a common mean of 179 apparently without regard to the concentration. Some work was done with untreated diseased juice. After making some preliminary tests a stock solution of equal parts of juice and water was used to prepare four other solutions each one-half as strong as the preceding. The five solutions, Series 15, were arranged in a Latin Square on five plants, and the set replicated four times. The total lesion counts were 1891, 1557, 1326, 806, and 900 and show evidence of the same leveling out of the dilution curve. The concentration of virus in this juice was not known.

Series 16 and 17 were made using a geometric ratio of two-thirds for the dilutions. The high counts at concentrations of 0.67 mg. per cc. and above show that the level portion of the curve does not correspond to the maximum values obtained with very concentrated preparations. Finally the last two series show for the Stanley crystals parallel performance of the two separate series, and the tendency for a maximum to exist in the neighborhood of 0.1 concentration.

TABLE II
LESION COUNTS OBTAINED WITH LATIN SQUARES

Latin square	Concentration—mg. per cc.					L. sq. totals
	.14	.12	.10	.08	.06	
1	375	850	751	815	481	3272
2	474	596	731	445	445	2691
3	571	537	596	513	438	2655
4	431	542	569	488	469	2499
Total	1851	2525	2647	2261	1833	11117

The results of an experiment conducted to show that solutions after dilution may give rise to more lesions than before dilution are given in Table II. The 0.14 solution was prepared by adding 2.00 cc. of stock containing 1.85 mg. per cc. to 24.44 cc. of water. The 0.06 solution was made by adding 1.00 cc. of the stock to 29.85 cc. of water. The intermediate concentrations were made by mixing three parts of the 0.14 and one part of 0.06, by mixing equal volumes, and by adding one volume of the 0.14 to three volumes of 0.06. The five solutions were applied to five plants in a Latin Square design. Four such Latin Squares were inoculated and the counts obtained from each square examined. In every case some one of the intermediate solutions gave greater counts than the extreme concentrations.

OTHER EXPERIMENTS WITH THE VIRUS CRYSTALS

The two stocks of virus crystals were compared by making up each lot in water solution and in phosphate buffer solution at each of two con-

centrations of the protein. The eight solutions were applied to 16 plants. A total of 1035 lesions were divided: 504, 531 on the basis of the source of the virus; 556, 479 on the basis of the media, the phosphate solutions giving the larger number; and 587, 448 for the contrast of the two concentrations. The protein isolated in this laboratory showed an infective capacity that was equivalent to the virus protein presented by Dr. Stanley.

An experiment was designed to determine whether it was important to control the amount of liquid on the rubbing cloth. Usually three or four half leaves were rubbed before rewetting the cloth. The cloth was not as wet for the last half leaf as for the first half leaf. By extending to ten the number of half leaves rubbed before rewetting the cloth the drying becomes very evident. To determine whether this influenced the lesion count, half leaves rubbed with a cloth which had just been wet were compared with half leaves which received later rubs. This was done by wetting a cloth with a solution containing 0.02 mg. per cc. of the virus and rubbing ten half leaves once each in turn. The process was repeated with a new cloth for a total of nine replications. The nine half leaves which received the first rub from a cloth constituted a group which could be compared with the nine which received the second rub and so on.

Rub No.	1	2	3	4	5	6	7	8	9	10	Av.
Lesions	50	66	55	89	85	77	91	89	88	80	77
Lesions	82	76	74	109	100	110	107	86	76	91	91

The lesion counts obtained in two different experiments show that it made very little difference whether the cloth was quite wet or only slightly wet. There is possibly some suggestion of higher counts with the later rubs probably because more care was required to wet the leaf as the cloth became less moist.

DISCUSSION

The difficulties encountered in determining the relative infectivity of virus preparations are sharply revealed when the several preparations are formed by dilutions from a stock solution. The very considerable heterogeneity of the test leaves, the seeming crudity of the inoculation procedure, and the fact that solutions still show infectivity after dilution 10,000-fold or more all led to the general employment of dilution on a logarithmic scale. This soon brought the concentration of the virus so low that on further dilution the lesion count and the dilution were directly proportional. Unfortunately at this point the number of lesions per leaf or plant becomes quite small and large numbers of leaves must be inoculated to give a total count of any useful size. At the very best, assuming Poisson's law, the standard deviation of the total is equal to the square root of the total, so that a count of 100 has a standard deviation of ten. Actually the error seems to be not less than twice the theoretical.

The advantage, therefore, that dilute solution have of giving counts directly proportional to the concentration is somewhat nullified by the small counts obtained. Very strong solutions may be diluted considerably before the lesion count begins to decrease. Somewhere between this part of the curve and the region of low concentrations would seem to be a favorable concentration at which to make comparisons. It was in this range of dilution that the present work attempted an intensive study. It was found that when the concentration was such that about half as many lesions were obtained as the maximum possible, further moderate dilution did not decrease the lesion count but often increased it. It would be practically impossible to detect this using the customary scale of ratio dilutions. In this work the dilution interval was made quite small. Ten or more dilutions were interpolated in a range of dilution spanned by two points in previous work. If the results of such small dilutions are to mean anything there must be evidence that the unchanged or increased lesion count is not merely uncontrolled variation in the counts arising from the method of inoculating or differences in the test leaves. To make certain that this was not the case the dilution series were often run in duplicate using two sets of plants. The counts from each set of plants constitute separate series. It was found that even with very small dilution intervals the dilution series kept step with one another in exhibiting maxima and minima. If the differences between dilutions for the same series were the result of the inoculation technique there should be erratic differences between corresponding dilutions from the two series. This is not the case. Rather, if one set of plants gives an appreciably lower total number of lesions than the other set, the counts for all the pairs of individual dilutions maintain the same order. There seems no alternative but to conclude that the data for the range of dilution showing a constant or even slightly increasing count really represent the performance of the solutions and are not chance phenomena.

In a recent paper, Wyckoff, Biscoe, and Stanley (5) have determined by means of ultracentrifugal analysis that solutions of the tobacco virus protein diluted to 0.5 mg. per cc. show a molecular dissociation. This was not observed in stronger solutions even after a long time. Solutions with less than 0.5 mg. of protein per cc. were not reported upon but it is possible that further dissociation takes place in that range. If so, it would furnish an explanation for the undiminished or increased infectivity upon dilution in that range provided the smaller units are capable of initiating lesions.

The advantages of the incomplete block arrangement are clearly evident in the agreement of the lesion counts of the duplicate series of dilutions. Several of the curves are based on only nine plants for the ten dilutions. This is a smaller number of plants than were often used in previous work to establish one point where the dilution interval was vastly

greater between points and differences correspondingly easier to establish. There is no doubt that the same procedure will show to similar advantage in comparing the infectivity of virus preparations that are not merely a series of dilutions but arise from different sources or have undergone different treatments. This work also shows that such comparisons should not be made at concentrations in the neighborhood of 0.1 mg. per cc. but at somewhat lower concentrations where the unusual behavior upon dilution is not a complicating factor.

SUMMARY

Data for 20 dilution series of tobacco-mosaic virus are given and show that in the neighborhood of 0.1 mg. of virus protein per cc. the virus solutions may be diluted without a corresponding decrease in lesions produced on the leaves of *N. glutinosa*. There is considerable evidence that there may be an increase in infectivity after moderate dilution. This was found to hold for virus protein from two different sources.

The method of incomplete blocks made possible the comparison of solutions that differed but little in concentration since consistent dilution curves were obtained with only nine *N. glutinosa* plants using an arithmetical series of dilutions in place of the more common logarithmic series.

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SELECTION OF EFFICIENT METHODS FOR SOIL SAMPLING

W. J. YOUDEN AND A. MEHLICH

INTRODUCTION

Since the purpose of any soil survey is to characterize the area as fairly as possible within the limitations of the number of samples that it is possible to take and analyze, the efficiency of the sampling plan adopted is a major concern. Soil sampling procedure may be divided into two parts: first, the allocation of the samples over the region under survey, and second, the technique of sampling. The details of sampling, in particular the description of the various horizons, have been worked out with care (2, 4). Various considerations such as the slope and other pertinent physical features of the terrain, as well as the cover crops, have also been stressed (5, 8, 9, 10). The need for taking replicate samples is well recognized (5, 7). There appears to be a lack of quantitative information regarding the disposition of the replicate samples. In order to secure data to test the adequacy of various sampling plans, two soil types were studied using a sampling pattern that would bring out the relative agreement of duplicate samples separated by predetermined distances.

The classification of soils presupposes that the distinguishing characteristics of any given type are fairly constant. The range of variation of any particular characteristic for a soil type is, in general, obscured by local variations due to incidental conditions that may exist and which are often unknown to the surveyor. Thus, for example, two areas separated by some miles may have average values for pH (determined by taking a great many samples) that differ by only two- or three-tenths. Individual samples within each area may show a far greater variation. Consequently, if but one sample is taken from each of these widely separated areas, the difference in pH between the samples reflects not only the real difference in acidity between the two areas but also the local conditions which may have been encountered. The sampling procedure adopted in this work permits the separation of these two factors and enables the investigator to determine the real range of variation of the soil character. Furthermore, it is possible to devise sampling schemes which will indicate whether differences found between separated areas are of a magnitude found naturally in the soil type or are so large as to constitute evidence that the soil has been altered as a result of exposure to contamination on a large scale.

REGIONS SURVEYED

Care was taken to select regions that would be as free as possible from complicating factors. This required that the sites be at a considerable dis-
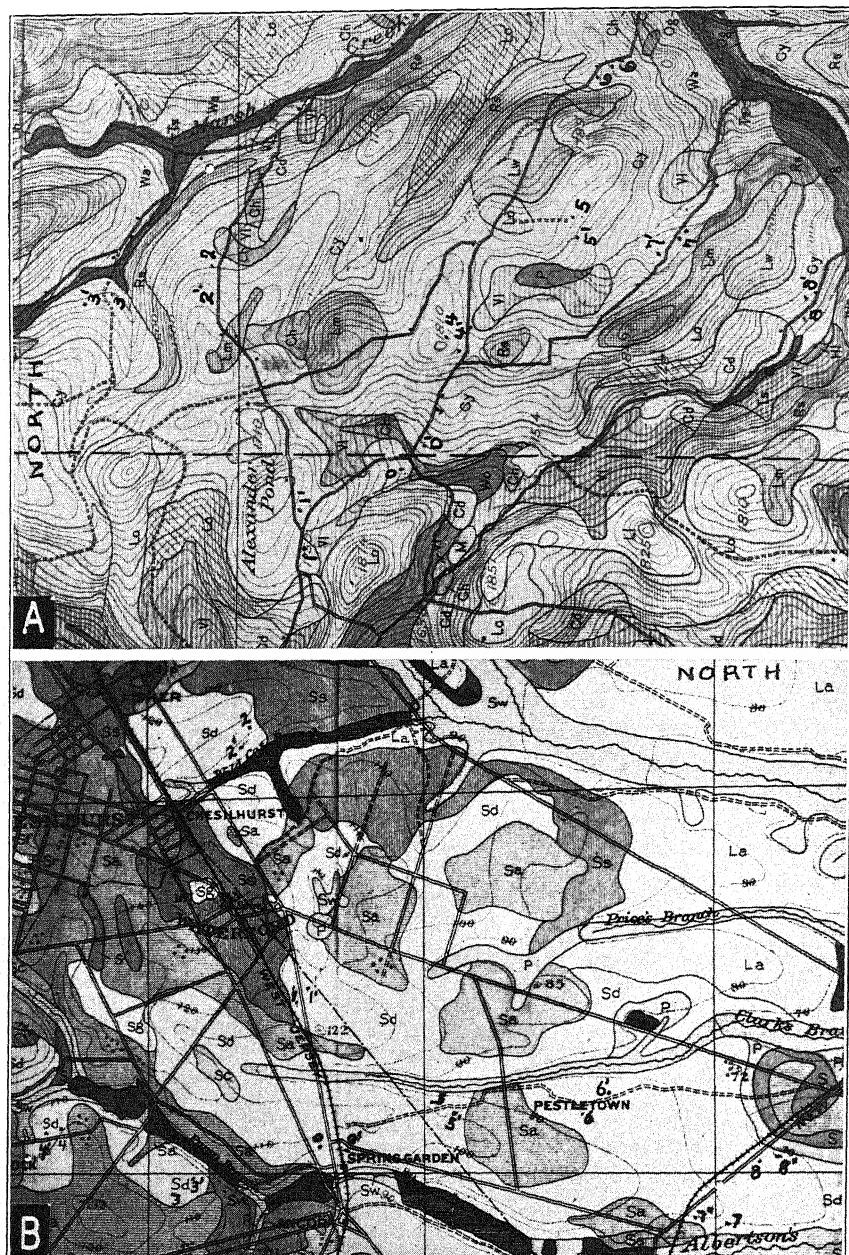
FIGURE 1. A. Section of soil survey map of Broome County, New York (6). B. Section of soil survey map of the Camden area, New Jersey (1) showing location of the places sampled. Scale 1 inch equals 1.3 miles.

tance from manufacturing centers. A region not under intensive cultivation was sought because of the manifest difficulty in securing samples in tilled areas which would not reflect the results of agricultural operations. It was also essential that recent soil survey maps be available as a guide in determining the soil type.

Figure 1 A shows a reproduction of portion (Lat. 42°05′, Long. 75° 32′) of the soil survey map for Broome County, New York (6). In this section the predominating soil is Culvers gravelly silt loam (Cy). The soil here is a dark-brown, friable, slightly gritty, silt loam to a depth of six or eight inches. Below this depth increased compactness and larger stones are encountered. It was found that a considerable portion had been set aside as a wild life sanctuary and that most of the remainder was not under cultivation. Furthermore a preliminary survey showed a marked uniformity in the cover crop of native grasses. Wild strawberries were invariably present at the sampling points. The samples were collected June 22, 1937.

The other area (Lat. 39° 42′, Long. 74° 50′) surveyed on July 30, 1937 is shown in Figure 1 B which is copied from the soil survey of the Camden area, New Jersey (1). The prevailing soil here is Sassafras loamy coarse sand (Sd). It is described in the soil survey (1, p. 13) as consisting "of brown, grayish-brown, or yellowish-brown loamy coarse sand about eight inches thick. This rests on reddish-yellow or yellowish-red coarse loamy sand, which in most places extends to a depth greater than three feet." This soil type in the area surveyed was not under cultivation. The cover varied from conifers to scrub oak, both being commonly present. Blueberries were always found in the areas sampled.

SAMPLING PROCEDURE

The same procedure of sampling was followed with both soil types. In each case nine stations were sampled with approximately one mile between adjacent stations. At each station two substations were selected one thousand feet apart. Two sampling areas one hundred feet apart were located at each substation. Finally, in each sampling area two sample points were taken ten feet apart. These distances were not adhered to rigidly and this combined with complete freedom as to direction permitted the exercise of the utmost discrimination in securing the samples under comparable conditions. At each sampling point three layers of soil were collected. These were, in the case of the Culvers soil, the top two inches, the two to six inch layer, and the top six inches or composite sample; and for the Sassafras the A_0, A, and B horizons. The A_0 horizon consisted of decomposed organic matter after removal of loose leaves and debris. The A horizon was sampled to the full depth, the B horizon to a depth of five inches.

The recording of samples was greatly facilitated by the adoption of a numbering system by which each sample was identified by a code number

of three digits, xyz. The digit in the x position is either o, 1, or 2 and these represent the top layer, lower layer, and the composite samples respectively for the Culvers loam, and the three horizons A_0, A, and B for the Sassafras sand. The nine stations were given the numbers o to 8 and these
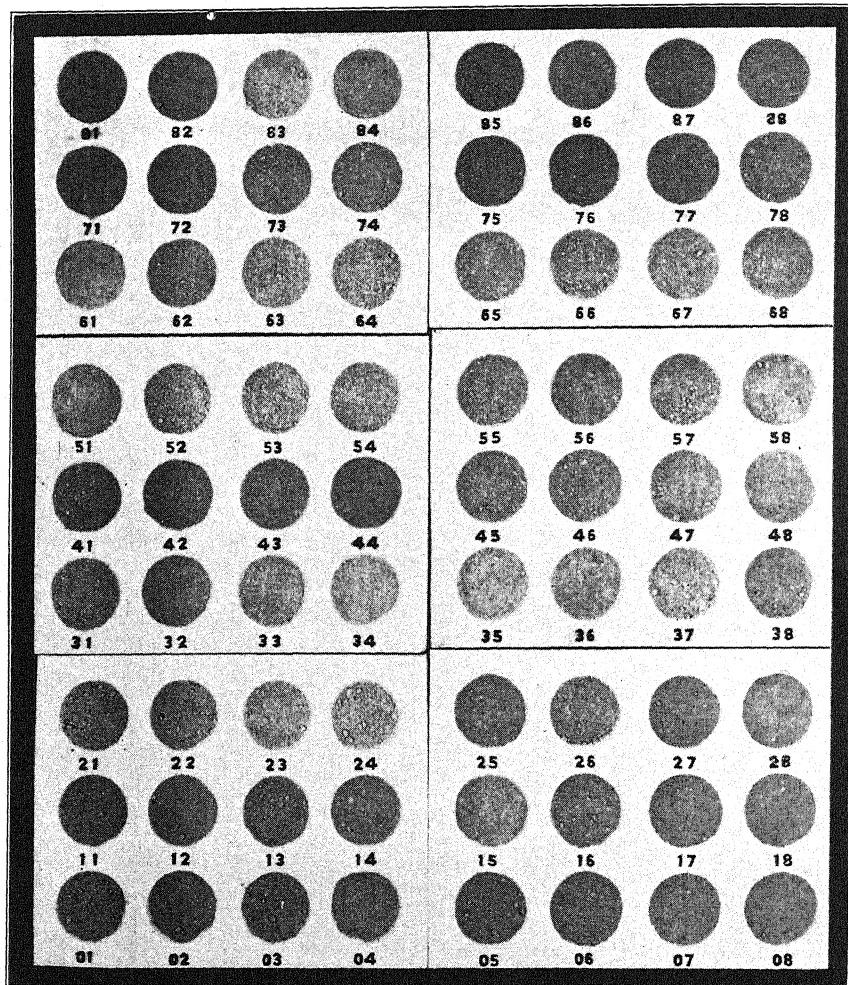
FIGURE 2. The 2–6 inch layer of Culvers gravelly silt loam displayed on spot plates. All the samples in a horizontal row are from the same station. Disposition of samples within station described in text.

appear in the y position in the code. The eight sample points comprising each station were numbered 1 to 8 and occupy the z position in the number.

On the maps are shown the locations of the two substations for each station. Sample points 1, 2, 3, and 4 make up one substation and 5, 6, 7,

and 8 the other substation one thousand feet away which is marked with a prime. The same convention holds for all stations. It is readily seen that the samples at the extreme north of the Culvers area (substation 3′) were numbered as follows:

0–2 inch layer	035	036	037	038
2–6 " "	135	136	137	138
Composite	235	236	237	238

Samples ending in 5 and 6 are taken ten feet apart and are separated by one hundred feet from those ending in 7 and 8 which are in turn ten feet apart. Samples ending in 1, 2, 3, and 4 follow the same pattern.

LABORATORY EXAMINATION OF SAMPLES

The appearance of these soil samples may be judged from Figure 2. Figure 2 shows a photograph of the 72 samples of the two to six inch layer

TABLE I

pH VALUES FOR THE 72 SAMPLES OF CULVERS GRAVELLY SILT LOAM

Station number		Sample number								Station average
		1	2	3	4	5	6	7	8	
0	Surface layer	5.16	5.06	5.55	5.48	4.86	4.86	5.04	4.96	5.12
1		5.23	5.01	5.57	5.30	5.37	5.11	5.08	4.99	5.21
2		5.28	5.08	5.01	5.13	5.45	5.53	5.45	5.53	5.31
3		5.01	4.89	5.15	5.01	4.72	4.89	4.87	4.72	4.91
4		4.52	4.49	4.62	4.35	4.91	4.89	4.71	4.82	4.66
5		4.28	4.25	4.38	4.59	4.62	4.76	4.86	4.81	4.57
6		4.77	4.77	4.93	4.91	4.74	4.87	4.84	4.76	4.82
7		4.79	4.59	4.82	4.72	5.01	4.71	4.77	5.04	4.81
8		4.96	5.11	4.87	4.43	4.94	4.55	4.49	4.64	4.75
									Av.	4.91
0	2″–6″ layer	5.21	5.18	5.31	5.37	4.84	4.81	4.87	4.87	5.06
1		5.13	4.99	5.30	5.16	5.28	5.18	5.04	5.08	5.15
2		5.25	5.11	5.08	5.09	5.15	5.16	5.20	5.28	5.17
3		4.86	4.84	4.93	4.87	4.84	4.82	4.86	4.84	4.86
4		4.52	4.60	4.60	4.37	4.91	5.11	4.77	4.86	4.73
5		4.40	4.45	4.65	4.93	4.71	4.79	5.15	4.84	4.75
6		4.87	4.69	4.94	4.89	4.72	4.71	4.86	4.81	4.82
7		4.82	4.55	4.67	4.74	5.01	4.82	4.60	4.84	4.76
8		5.13	5.09	4.69	4.60	4.81	4.32	4.54	4.64	4.73
									Av.	4.89
0	Composite	5.18	5.16	5.20	5.26	4.77	4.74	4.81	4.86	5.00
1		5.09	4.87	5.28	5.09	5.20	5.08	5.04	4.98	5.09
2		5.20	5.08	5.04	5.01	5.28	5.30	5.25	5.40	5.20
3		4.89	4.81	5.11	4.86	4.69	4.76	4.79	4.79	4.84
4		4.49	4.49	4.57	4.37	4.84	4.87	4.71	4.79	4.65
5		4.30	4.35	4.45	4.69	4.59	4.69	4.98	4.71	4.60
6		4.74	4.64	4.86	4.82	4.59	4.84	4.87	4.72	4.77
7		4.82	4.45	4.69	4.74	4.87	4.72	4.69	4.91	4.74
8		4.99	5.09	4.77	4.54	4.89	4.43	4.40	4.49	4.71
									Av.	4.84

of Culvers loam arranged on porcelain spot plates. The numbers identify the station and sample number. Although the colors are not reproduced the different shades show something of the color range exhibited by the soils. This device affords an excellent means of comparing a large number of soil samples as regards their physical appearance.

TABLE II

pH VALUES FOR THE 72 SAMPLES OF SASSAFRAS LOAMY COARSE SAND

Station number		Sample number								Station average
		1	2	3	4	5	6	7	8	
0		4.21	4.16	4.32	4.23	4.08	4.20	3.94	4.57	4.21
1		4.40	4.47	4.40	3.98	4.06	3.53	4.15	3.76	4.09
2	Surface layer	3.93	3.57	3.64	4.20	4.23	4.94	3.83	3.93	4.03
3		3.89	3.96	4.13	4.01	3.89	3.83	3.74	3.88	3.88
4		3.86	3.88	3.98	3.74	4.13	4.27	4.16	4.03	4.01
5		3.89	3.86	3.39	3.54	3.69	3.52	4.01	4.15	3.76
6		4.21	3.91	3.94	4.16	3.81	3.79	3.84	3.50	3.90
7		3.57	4.33	4.11	3.71	3.74	3.67	3.94	3.81	3.86
8		3.56	3.57	3.69	3.56	4.05	3.74	3.72	3.69	3.70
									Av.	3.94
0		4.45	4.38	4.43	4.54	4.47	4.54	4.62	4.52	4.49
1		4.50	4.49	4.35	4.28	4.20	4.26	4.40	4.13	4.33
2	A horizon	4.11	3.68	4.21	4.16	4.33	4.65	4.20	4.67	4.25
3		4.23	4.38	4.55	4.55	4.47	3.88	3.88	3.94	4.24
4		4.06	4.10	4.35	4.18	4.67	4.86	4.43	4.64	4.41
5		4.20	4.18	3.89	3.96	4.06	3.95	4.43	4.35	4.13
6		4.64	4.55	4.64	4.55	4.20	4.06	4.21	3.96	4.35
7		4.32	4.51	4.32	4.23	4.23	4.38	4.30	4.40	4.34
8		3.93	4.21	3.91	3.96	4.47	4.43	4.47	4.01	4.17
									Av.	4.30
0		4.77	4.74	4.99	4.84	4.71	4.69	4.72	5.18	4.83
1		4.67	4.45	4.64	4.65	4.71	4.30	4.52	4.49	4.55
2	B horizon	4.74	4.54	4.67	4.67	4.82	4.91	4.67	4.84	4.73
3		4.74	4.59	4.77	4.60	4.59	4.60	4.38	4.55	4.60
4		4.40	4.47	4.62	4.57	4.93	4.89	4.74	4.57	4.65
5		4.42	4.81	4.60	4.49	4.64	4.59	4.93	4.69	4.65
6		5.30	5.08	4.72	4.49	4.60	4.69	4.62	4.64	4.77
7		4.67	4.67	4.65	4.71	4.67	4.62	4.67	4.69	4.67
8		4.50	4.49	4.33	4.35	4.49	4.49	4.65	4.49	4.47
									Av.	4.66

The acidity is only one of many soil properties but its ease of measurement was an advantage in obtaining information regarding sampling methods. The acidity was determined by means of a Leeds and Northrup glass electrode. In each day's run one complete set of 72 samples all from the same horizon were measured. The measurements were repeated for one set and the standard deviation of a single measurement found to be 0.05 pH unit. The soil suspensions were prepared by stirring together 50 cc. of

water and 20 grams of soil, which had been air-dried and put through a 10 mesh sieve. The mixture was stirred occasionally during an interval of 30 minutes before determining the acidity.

The complete list of pH values for the Culvers loam is given in Table I and for the Sassafras sand in Table II together with the mean values for the various stations. Each table is divided into thirds corresponding to the three layers sampled. The eight values in any row are from the same station, in accordance with the numbering scheme described above. The immediate impression given by the data is that the averages of the several stations for any set agree more closely than individual samples from the

TABLE III

DIFFERENCE IN pH OF DUPLICATE SAMPLES OF CULVERS GRAVELLY SILT LOAM

Distance between duplicate samples	0–2 inch layer		2–6 inch layer	
	Av. diff.	Max. diff.	Av. diff.	Max. diff.
10 feet	0.14	0.44	0.11	0.49
100 feet	0.18	0.84	0.20	0.53
1000 feet	0.26	0.69	0.25	0.81
1–3 miles	0.36	1.32	0.28	1.05

TABLE IV

DIFFERENCE IN pH OF DUPLICATE SAMPLES OF SASSAFRAS LOAMY COARSE SAND

Distance between duplicate samples	A_0 horizon		A horizon		B horizon	
	Av. diff.	Max. diff.	Av. diff.	Max. diff.	Av. diff.	Max. diff.
10 feet	0.23	0.76	0.17	0.59	0.13	0.46
100 feet	0.27	1.11	0.19	0.59	0.18	0.81
1000 feet	0.31	1.37	0.28	0.99	0.19	0.70
1–3 miles	0.35	1.55	0.28	1.18	0.20	1.00

same station, although samples separated by but ten feet show very good agreement. The average difference between samples separated by 10 feet, 100 feet, 1000 feet, and by stations were calculated from the data and are listed in Tables III and IV. The maximum differences encountered are also listed. The average differences become larger as the distance between the duplicate samples increases. In the case of the lower layers the average difference is evidently approaching a limiting value. This is shown in Figure 3 which brings out the greater variability of the surface layers in comparison with the lower layers. The differences do not arise to any important extent from the error of analysis, since the correction for this source amounts to less than 0.02 pH unit.

STATISTICAL ANALYSIS OF DATA

An elegant analysis of these data is furnished by the partition of the variance into several categories. This is given in detail for the surface layer of the Culvers loam in Table V. The z distribution (3, p. 248) shows that statistical significance may be attached to the differences in variation, i.e., the mean squares, calculated for the several items. The data may be con-
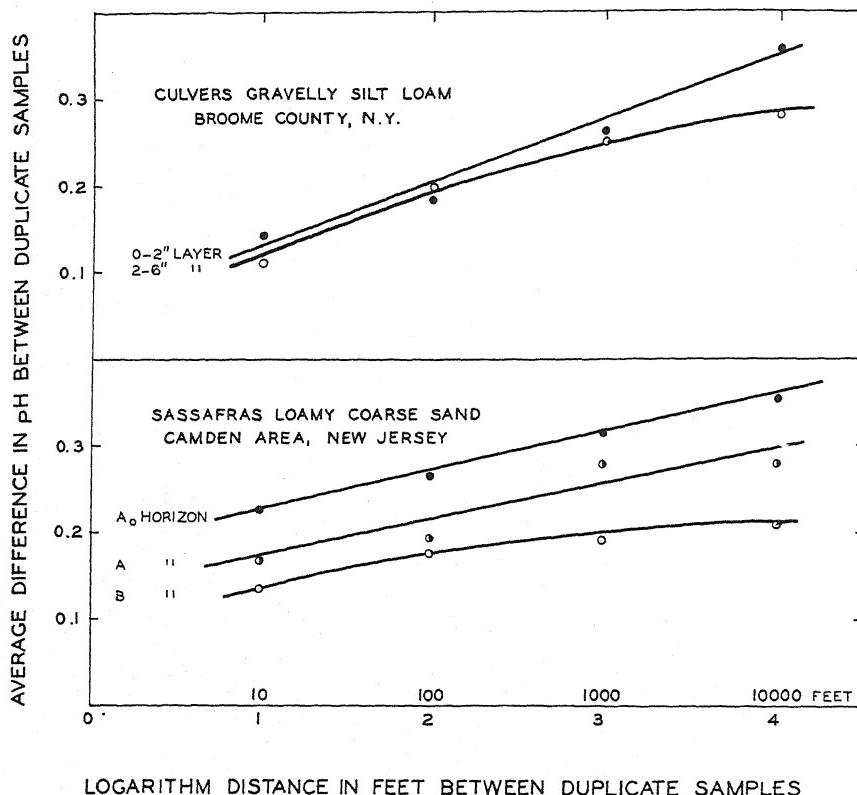
FIGURE 3. Graph showing the relative variability of the various soil layers. The average difference between duplicate samples becomes larger as the linear distance between the samples increases. The curves show that this difference approaches a maximum value for the lower layers.

sidered as 36 pairs of duplicate samples (ten feet apart) and Student's method used. The mean square in that case will be twice as large as that given for sample points in Table V. This is the case because Student's method gives the variance of the difference between two samples while the value in the table is the variance of a single sample. The values for each pair of sample points may be combined giving 36 items which may be ar-

ranged in 18 pairs of neighboring sample areas. Student's method may be applied to these values in turn, and the result compared with the entry after sample areas.

TABLE V

ANALYSIS OF VARIANCE OF THE SURFACE LAYER OF CULVERS GRAVELLY SILT LOAM

Item	Degrees freedom	Sum of squares	Mean square	Standard deviation
Between sample points	36	0.5758	0.01599	0.126
Between sample areas	18	0.6536	0.03631	
Between substations	9	1.3393	0.1488	
Between stations	8	4.0984	0.5123	
Total	71	6.6671		

The mean squares for the other five sets are tabulated in Table VI. In only one instance, the A horizon of Sassafras sand, does the mean square fail to increase as the distance increased between places contrasted. The mean squares for substations and stations in this column do not differ significantly.

TABLE VI

MEAN SQUARES FROM ANALYSIS OF VARIANCE

Variance between	Culvers loam		Sassafras sand		
	2–6" layer	Composite	A_0 horizon	A horizon	B horizon
Sample points	0.01176	0.01391	0.04618	0.02225	0.01409
Sample areas	0.03471	0.02494	0.06546	0.03621	0.03519
Substations	0.11432	0.11856	0.15019	0.21380	0.04982
Stations	0.2724	0.3441	0.21586	0.10684	0.09536

Following a procedure given in detail by Tippett (11, p. 92, 93) the estimated variance for the different items has been calculated. These are as follows for Culvers gravelly silt loam:

	0–2 inch layer	2–6 inch layer
Sample points	0.01599	0.01176
Sample areas	0.0101	0.0115
Substations	0.0281	0.0199
Stations	0.0454	0.0198

From these may be calculated the variance of the station mean of eight samples. First two sample points are averaged, the result having a variance of 0.01599/2, or 0.0080. This is added to 0.0101 to give the variance for a sample area as sampled, or 0.0181. Two of these constitute a substation so that a substation as sampled has a variance of 0.0181/2 + 0.0281, or 0.0372. The value of 0.0640 is obtained for the station variance in the same way.

This checks the value taken from Table V since the variance of a station mean is one-eighth of 0.5123, or 0.0640.

The values are now in hand to compute the variance of station means when sampled according to some other pattern. Thus if but four samples are taken at intervals of 1000 feet the variance is

$$\frac{0.0160+0.0101+0.0281}{4}+0.0454=0.0590$$

or less than that obtained with eight samples most of which are taken in close proximity to each other. Any other combination of distances may be assumed and the efficiency of the arrangement determined.

DISCUSSION

The usefulness of laboratory examination of soil samples in the classification of soil types depends on the selection for study of soil properties which vary over a limited range within the type and which take on uniquely different values for different types. The range within which a given property may vary for each type may be determined by choosing a number of localities all classified as the same type and sampling very intensively by taking a great number of samples. The local irregularities will disappear in the station averages which may then be used to estimate the range over which the property varies. This is equivalent to making the first term in the computation in the preceding paragraph approach zero since the denominator represents the number of samples taken. The remaining quantity, 0.0454, which characterizes the station variance may be doubled to give the variance of the difference between two stations. The standard deviation is the square root of this quantity, or 0.30, and when divided by 1.253 gives the average difference, or 0.24. Differences up to two and one-half times the average difference will be frequently encountered so that the range within which the great bulk of the stations will fall is 0.6 pH unit. The maximum difference found between any pair of the nine station means, even with limited sampling, is 0.74. Similar calculations for the lower layer give an expected range of about 0.4 pH unit. The maximum observed difference between station means is 0.44. In the instance of the two soil types under consideration there is a marked difference in the average values for the acidity.

The problem, referred to in the introduction, of distinguishing whether differences in properties found between widely separated areas are such as occur naturally in the type or are indicative of the superimposition of unusual conditions, also requires a knowledge of the variation found within the type. In some cases soils have been exposed to industrial waste gases, such as sulphur dioxide, and it becomes important to ascertain whether

the soil has been damaged, and if so, the limits of the area affected. It should be clear that supposed differences in properties must be considered in view of the distances involved. Certainly the evaluation of such differences in terms of the agreement of samples taken within a limited area courts misinterpretation.

A recent article by Walker (12) discusses the general question of heterogeneity within a soil type and its bearing on field experiments. This author strongly advocates the use of statistical control in soils experiments. Soil studies are often carried on simultaneously with yield trials. The sampling methods used in the soil work may properly be given the same consideration that is now directed to the design and analysis of the yield trials.

The evidence in this work indicates that the sampling of large areas may have been inefficient because the replicates were taken too near to each other. This may be demonstrated by eliminating all the even or all the odd numbered samples in Tables I and II and noting that the stations are nearly as well defined by the remaining values. Samples taken close together too often show the influence of the same local situation and contribute little more than one sample would to the formulation of an accurate estimate of the whole area. When the samples are more widely dispersed there is far greater opportunity for the transient conditions to neutralize themselves rather than perpetuate themselves in the average for the area.

SUMMARY

Culvers gravelly silt loam from Broome County, New York, and Sassafras loamy coarse sand from the Camden area, New Jersey, were sampled at nine stations scattered over an area of several square miles. At each station samples were collected at definite intervals and the acidity of the samples determined. The results show in each case that samples from widely separated points vary more than samples taken close together. This was also observed to hold for the lower horizons where the variation was not as great and tended to reach a maximum value characteristic of the soil type.

The data were subjected to statistical analysis to show the relative efficiency of various spacings for replicate samples when large areas are surveyed. It was found that intervals as low as 10 feet, or 100 feet, were too small to constitute an effective method for sampling these areas.

The sampling procedure was discussed with reference to its application in crop fertility studies, soil classification, and the investigation of possible damage to soils over large areas.

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GERMINATION AND SEEDLING PRODUCTION
OF ARCTOSTAPHYLOS UVA-URSI

JOHANNA GIERSBACH

INTRODUCTION

The species *Arctostaphylos Uva-ursi* (L.) Spreng. (bearberry) is hard to propagate from seed as the fruits may lie on the ground for years before germinating. This difficulty is caused by a dormant embryo combined with an unusually hard fruit coat. Many rosaceous forms, as reported by Crocker and Barton (2) and Giersbach (4), and also other forms such as *Symphoricarpos racemosus* (3), *Tilia americana* (1), and *Halesia carolina* (5), presented similar problems. In such cases the hard coat of either nut or seed must be destroyed by mechanical or chemical means, or by soil organisms (6), which act on fruit and seed coats during a warm season. After the coats had been made permeable the embryos after-ripened and germinated readily after a period at low temperature.

The fruit of the genus *Arctostaphylos* is a drupe, where the pericarp consists of a fleshy outer layer and the hard inner stone. The latter is composed of 10 nutlets and may contain from 1 to 10 seeds. The Rancho Santa Ana botanic garden of native plants of California, Herbarium, botanical library (7, p. 14) gives a general description of the species of *Arctostaphylos* as follows: "The seed of the varieties where the fruit breaks up into small nutlets germinates most readily and generally germinates the first year. But those where the nutlets are combined in a large solid nut do not germinate until the second and third year and then the germination is very poor." *Arctostaphylos Uva-ursi* belongs to the latter group.

Arctostaphylos Uva-ursi grows abundantly along the Long Island highways and seed material was collected and furnished through the courtesy of Mr. C. J. MacGregor, Nursery Superintendent of the Long Island State Park Commission. The fruits were freed from pulp by use of a Hobart machine. During this process some of the stones fell apart into single nutlets or stone-pieces, while others remained a solid stone.

Cutting tests showed that entire nutlet stones contained from 1 to 9 embryos each; 100 of such stones contained 350 to 415 embryos. The average number of embryos in 100 stone-pieces varied according to the size of the piece; 100 single nutlets contained between 67 and 85 good embryos, depending upon the crop. Germination and seedling production percentages reported in this paper are based on the approximate number of good embryos to 100 nutlet stones, 100 stone-pieces, or 100 single nutlets.

EXPERIMENTAL RESULTS

During the following experiments entire stones, stone-pieces, or single nutlets were treated with concentrated sulphuric acid to break down the stony coats. This treatment was followed by high temperature application, preceding low temperature in which the embryos were to after-ripen.

TABLE I

EFFECT OF CONCENTRATED SULPHURIC ACID AND TEMPERATURE TREATMENTS ON 1933 CROP OF ARCTOSTAPHYLOS UVA-URSI

Seed material	Acid treatment (hours)	°C. for 15 months	Per cent germination after months in peat at low temperatures						Per cent seedling production after 1 winter in board-covered frame		Total percentage of germinated seeds	
			4		8		12					
			a	b	a	b	a	b	a	b	a	b
Entire stones	None	5	0	0	0	0	0	0	1	0	1	0
		10	0	0	0	0	0	0	0	0	0	0
	1	5	0	0	0	0	0	0	3	7	3	7
		10	0	1	0	1	0	2	3	12	3	14
	2	5	0	1	0	1	1	1	7	14	8	15
		10	1	0	1	1	2	1	16	20	18	21
	3	5	0	1	1	2	2	2	19	21	21	23
		10	1	2	1	3	3	4	22	17	25	21
	4	5	0	5	1	7	1	7	23	25	24	32
		10	1	6	2	10	3	10	17	22	20	32
Single nutlets	None	5	0	1	0	1	0	3	10	5	10	8
		10	0	0	0	0	0	1	5	1	5	2
	0.5	5	0	1	0	3	0	3	7	10	7	13
		10	0	0	0	3	0	4	22	16	22	20
	1	5	1	4	1	4	3	5	19	19	22	24
		10	1	7	3	11	4	12	27	22	31	34
	2	5	1	7	1	10	1	11	23	22	24	33
		10	3	5	5	11	8	12	24	24	32	36
	3	5	3	18	5	26	5	26	26	18	31	43
		10	8	12	12	20	15	22	39	24	54	46
	4	5	3	15	4	22	5	22	32	15	37	37
		10	5	15	10	27	11	28	15	18	26	46

a = No period at 20°C. preceding periods at 5° or 10° C.
b = Two months at 20° C. preceding periods at 5° or 10° C.

For oven tests, the seed material was placed in moist granulated peat moss in containers that were kept in electrically controlled ovens. For plantings in flats, entire stones or single nutlets were planted in soil containing peat, sand, and soil in equal parts. The flats were kept in various conditions.

Oven tests. Cleaned entire stones and single nutlets were treated with concentrated sulphuric acid for various lengths of time, mixed with moist granulated peat moss and kept at 5° C. and 10° C. with and without a preceding two-month period at 20° C. Table I shows that the entire stones as well as the single nutlets scarcely germinated without preceding acid treatment. A four-hour application of sulphuric acid was more effective than one, two, and three hours for entire stones. Three hours, however, was sufficient for single nutlets. Germination percentages did not exceed 10 for the entire stones and 28 for single nutlets. This may have been due to the fact that the thickness of coats varied and the acid treatment was not equally effective. Some of the coats of nutlets or entire stones were destroyed to the point of seed injury, while others were not treated enough. The entire stones or single nutlets, where the coats were effectively removed, germinated after a period of four to eight months in the cold, 10° C. and 5° C. being equally good for after-ripening. Germination was considerably higher at low temperature with a preceding two-month period at 20° C. When germination had entirely ceased after 15 months at low temperature, the seed material of this experiment was planted in flats in June, exposed to the summer heat, and wintered in a board-covered frame. The following spring a considerable number of seedlings appeared in the case of acid-treated seeds, while germination of untreated ones remained low (Fig. 1, I and Table I). The importance of the length of the high temperature period preceding low temperature when combined with treatment of the seed material with concentrated sulphuric acid was especially noticeable in the 1935 crop when germination in moist granulated peat moss at 10° C. was recorded (Table II).

TABLE II

EFFECT OF CONCENTRATED SULPHURIC ACID AND PRECEDING PERIODS AT 25° C. ON GERMINATION IN MOIST GRANULATED PEAT MOSS AT 10° C.

Seed material	Acid treatment (hours)	Per cent germination after months at 10° C.														
		5					10					15				
		Preceding months at 25° C.														
		N*	2	3	4	5	N*	2	3	4	5	N*	2	3	4	5
1934 crop. Small stone-pieces	1	6	6	7	10	10	13	12	13	22	20	15	12	18	22	20
	2	10	14	10	14	25	20	24	21	30	31	22	25	35	33	31
	3	8	16	8	13	18	17	37	22	29	27	19	38	34	37	27
	4	6	8	10	7	9	11	28	21	19	27	13	29	28	21	27
1935 crop. Single nutlets	None	0	0	0	1	1	0	1	1	2	2	1	1	1	2	4
	1	0	0	0	0	0	1	0	1	1	4	1	0	1	1	4
	2	2	7	4	11	9	6	12	12	15	15	8	13	15	18	19
	3	2	6	4	5	11	8	14	13	11	21	8	15	15	17	26
	4	2	9	6	12	20	8	19	17	26	33	14	22	20	33	35

* None.

Samples of 100 single nutlets each were planted in the greenhouse monthly from lots in moist peat moss at 5° C. and 10° C., with preceding periods of one, two, and four months at 20° C. or 30° C. during a period
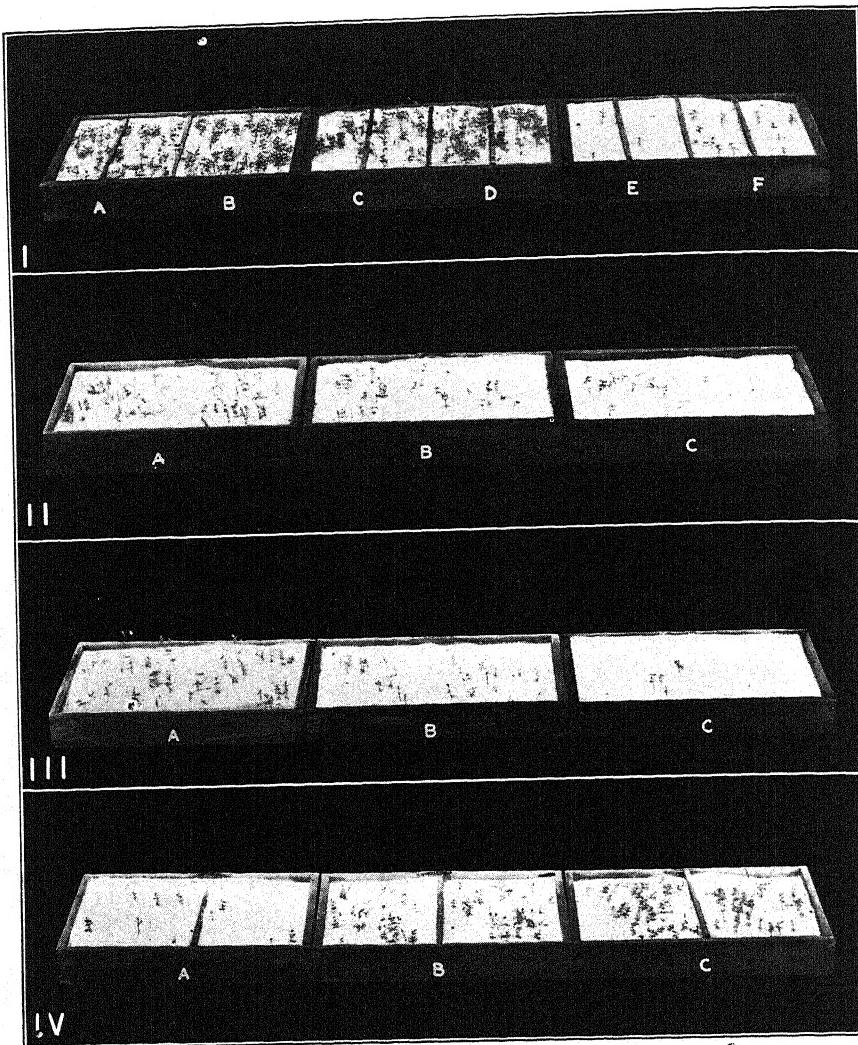
FIGURE 1. Seedling production of *Arctostaphylos Uva-ursi*. I. In spring of 1936, planted June 1935 following 15 months in moist peat moss at 5° and 10° C. (A) 1 hour concentrated H_2SO_4; (B) 2 hours; (C) 3 hours; (D) 4 hours; (E) no acid; (F) no acid, sand instead of peat. II and III. Effect of planting time in 1934 on seedling production in spring of 1935. II. Entire stones, 3-hour treatment of concentrated H_2SO_4. III. Single nutlets, 2 hours concentrated H_2SO_4. (A) January; (B) April; (C) August. IV. Effect of lengths of treatment with concentrated H_2SO_4 on entire stones. (A) No acid; (B) 2 hours; (C) 4 hours.

of 12 months. Since no seedlings appeared during a period of two months in the greenhouse, these sample plantings were alternated monthly between the 10° C. room and the greenhouse, but still no seedling production occurred.

Alternating low temperatures (3° to 10°, 5° to 10°, and 5° to 15° C. alternated twice monthly) were also tried for after-ripening but were found to offer no advantage over constant low temperatures.

That the embryos of *Arctostaphylos Uva-ursi* were not very dormant was shown when the hard coats were removed by cracking fruits open and placing the seeds in moist granulated peat moss. In this case, germination began after two months at constant temperatures of 5° or 10° C., as well as weekly alternating temperatures of 1° to 10°, 5° to 10°, and 10° to 30° C., and was concluded after six months (Table III). Various other temperatures were tried but gave negative results. As it was difficult not to injure the seeds while cracking the coats, only small seed samples were available.

TABLE III

EFFECT OF REMOVING COATS ON GERMINATION OF ARCTOSTAPHYLOS UVA-URSI AT VARIOUS TEMPERATURES. 1932 CROP

Temp. ° C.		Per cent germination after months in peat*				
		2	3	4	5	6
5		0	8	32	32	32
10		4	16	24	28	28
1 to 10	Weekly alternation	0	4	16	20	28
5 to 10		12	20	20	20	32
10 to 30		0	0	1	8	12

* Percentage based on samples of 25 seeds.

To test the relative effectiveness of soil and granulated peat moss samples of single nutlets pretreated with concentrated sulphuric acid for 1.5 hours were mixed with each medium and placed at 10° C., preceded by periods of various lengths at 25° C. Table IV shows that soil was inferior to peat.

Flat plantings. Entire stones and single nutlets were planted in flats in the soil mixture described above. Plantings were made on the fifteenth of each month throughout the year 1933, and in December 1932. These flats were wintered outside in open, mulched, or board-covered cold frames. The open frame seemed to be less favorable for seedling production of *Arctostaphylos Uva-ursi*, while the board-covered and mulched frames were equally good. Very few entire stones germinated after the first winter, and not many more after the second and third winters. Seedling production of single nutlets was somewhat higher, as probably less stony coat substance had to decay to enable the seed to after-ripen. The

percentage of seedlings did not increase much after the third winter. It is evident from the results of the spring and summer plantings that high temperature alone as pre-treatment was not sufficient to assure a good crop of seedlings after a cold period.

TABLE IV

COMPARISON OF GRANULATED PEAT MOSS AND SOIL AS MEDIA FOR GERMINATION OF SINGLE NUTLETS AT 10° C. NUTLETS TREATED FOR 1.5 HOURS WITH CONCENTRATED SULPHURIC ACID. 1934 CROP. SAMPLES OF 200 USED FOR EACH TEST

Pre-treatment at 25° C. (months)	Per cent germination after months at 10° C.					
	6		12		18	
	Peat	Soil	Peat	Soil	Peat	Soil
None	16	3	28	7	31	7
2	10	9	36	19	40	21
3	13	7	35	10	36	12
4	12	3	28	3	30	3
5	22	7	31	7	32	7

Entire stones, stone-pieces, and single nutlets were treated with concentrated sulphuric acid for various lengths of time and were planted in flats in June 1935 (Table V). The percentage of seedling production in the spring of 1936 was as high as 65 to 76 for entire stones with an acid treatment of three, four, and five hours. Stone-pieces treated for four and five hours gave 61 and 57 per cent. The optimum time for treating single nutlets seemed to be three hours. A longer acid treatment reduced seedling production of single nutlets.

TABLE V

SEEDLING PRODUCTION IN FLATS WINTERED IN A MULCHED FRAME AFTER TREATMENT WITH CONCENTRATED SULPHURIC ACID. 1934 CROP. PLANTINGS MADE JUNE 7, 1935

Acid treatment (hours)	Per cent seedling production, spring 1936		
	Entire nutlet stones	Stone-pieces	Single nutlets
None	12	12	9
1	16	14	23
2	39	31	37
3	70	40	45
4	65	61	32
5	76	57	—

Table VI shows the results obtained from flat plantings made at intervals throughout the year 1934. Entire stones and single nutlets were treated with concentrated sulphuric acid for two and three hours, planted in flats, and mulched during winter. Seedlings were recorded in the spring of 1935, 1936, and 1937. For untreated entire stones, as well as for untreated nutlets, seedling production was poor. When treated with con-

centrated sulphuric acid for three hours, the January and April plantings of entire stones gave a fair amount of seedlings after the first winter (Fig. 1, II, A and B). In the case of plantings later than July, most seedlings came up after the second winter. Seedling production increased little after the third winter. Single nutlets treated with concentrated sulphuric acid for two hours before planting gave similar results (Fig. 1, III). However, the latter could be planted as late as July and still give a fair crop after

TABLE VI

EFFECT OF TIME OF PLANTING ON SEEDLING PRODUCTION OF ENTIRE NUTLET STONES WINTERED IN A MULCHED FRAME. 1933 CROP. PLANTINGS MADE ON FIRST DAY OF EACH MONTH

Seed material	Time of planting (1934)	No acid treat.			2 hrs. acid treat.			3 hrs. acid treat.		
		Per cent seedling production in spring								
		1935	1936	1937	1935	1936	1937	1935	1936	1937
Nutlet stones	January	1	3	—	13	26	—	14	18	—
	April	1	2	3	—	—	—	10	17	18
	May	1	2	—	—	—	—	—	—	—
	June	1	4	—	—	—	—	—	—	—
	July	0	1	1	3	16	23	9	30	32
	August	1	2	—	2	7	—	5	16	—
	September	0	4	—	1	14	—	1	7	—
	October	0	0	1	0	5	10	1	8	14
	November	0	—		0			1		
Single nutlets	January	1	3	—	16	24	—	35	41	—
	April	1	3	—	19	24	—	24	26	—
	May	3	5	—	—	—	—	—	—	—
	June	1	3	—	—	—	—	—	—	—
	July	3	4	4	18	24	27	26	28	28
	August	1	4	—	7	12	—	15	16	—
	September	1	3	—	5	15	—	8	20	—
	October	1	4	7	4	22	34	5	26	26
	November	1	—	—	1	—	—	4	—	—

one winter. Plantings in August and later produced the main seedling crop after the second winter. The importance of the length of the acid treatment for seedling production of entire stones planted in January is shown in Figure 1, IV.

Stone-pieces treated with sulphuric acid for two, three, and four hours were planted in flats and placed at 10° C. constant, or with one, two, and three-month periods at 21° C. When transferred to 21° C. after two, three, four, and five months at 10° C. scarcely any seedling production occurred in the greenhouse. However, when these flats were mulched over winter, a considerable number of seedlings came up the following spring.

To determine the effect of a salt solution on the coat, entire stones were alternated between soaking either in a saturated NaCl solution, in this solution diluted to 50 and to 10 per cent, or in tap water, or were exposed to freezing and thawing in these solutions over a period of three months.

At that time the stones had softened to the extent that they could be cut into separate nutlets. The stones so treated were planted in flats in March and gave not more than 3 per cent seedling production after one winter in a mulched frame.

Preliminary work on *Arctostaphylos patula* (so called by the California Forest and Range Experiment Station, Berkeley, California) showed this species to be similar to *Arctostaphylos Uva-ursi*, in requirements for germination.

SUMMARY

1. The stony fruits of *Arctostaphylos Uva-ursi* fail to germinate readily for two reasons—a hard coat and a dormant embryo.

2. The hard coat of entire stones, stone-pieces, and nutlets may be partially removed by treatment with concentrated sulphuric acid for various periods. Such treatment may be made more effective by a subsequent high temperature period in moist granulated peat moss.

3. After the coat problem is solved, low temperature is needed to overcome the dormancy of the embryo. Stone-pieces treated with concentrated sulphuric acid for three hours and then mixed with moist granulated peat moss and kept at 25° C. for two months, gave four per cent germination when transferred to 10° C. Single nutlets soaked in concentrated sulphuric acid for 90 minutes germinated up to 40 per cent with the same temperature treatment.

4. When entire stones were treated with concentrated sulphuric acid for 3, 4, or 5 hours, planted in flats on June 7th, and subsequently wintered in a mulched frame, seedling production amounting to 70, 65, and 76 per cent was obtained. Under the same planting conditions as for entire stones, stone-pieces and single nutlets gave 61 and 37 per cent seedling production after being subjected to acid treatments for 4 and 2 hours.

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GERMINATION AND SEEDLING PRODUCTION
OF SPECIES OF VIBURNUM

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INTRODUCTION

Seedling production of the genus *Viburnum* has long been a problem. It has been discussed in the literature without any definite and satisfactory answer. In 1894 Jack (5) reported "about seeds with a hard bony covering," which might commonly be expected to grow in the second rather than in the first year, especially if not planted until spring. He mentioned *Viburnum* as one of these. Pammel and King (8) collected the fruits of *Viburnum lantana*, stored them out-of-doors on the surface of the ground during the winter, and planted them in the greenhouse in the spring where one seedling was produced. They obtained no germination from *Viburnum opulus* wintered out-of-doors. Mitchell (6) germinated seeds of *V. acerifolium* and *V. prunifolium* on filter paper at 20° to 25° C. in light and dark. She obtained high germination percentages. Davis (4) observed two stages of germination of the highbush cranberry. The first was the growth of the radicle at a temperature of 68° F. or higher, the second the development of the cotyledons, still covered by the seed coat at a cold temperature of 40° to 50° F., during which time the root system would enlarge. After the cold period, seedlings would develop normally in a warm greenhouse. In 1927 Adams (1) reported on experiments with *V. lantana* and *V. pubescens* when seeds, freed from pulp, were planted in soil and were kept out-of-doors during the winter and placed in the greenhouse in the spring or kept in the greenhouse all the time. He also stored seeds dry in a tin box outside or at room temperature over winter. The latter were planted in soil in the greenhouse in the spring. Only two seedlings were produced in the case of *V. pubescens*. Sixty-five per cent seedling production was obtained for the cultures of *V. lantana* with cold treatment, 25 per cent in constant greenhouse. Very low seedling production was reported for dry-stored seeds. No greenhouse temperatures were given in this report. In 1934 Nichols (7) conducted germination tests with seeds of 200 species, where seeds, freed from pulp, were planted in the soil in the fall and placed in a greenhouse immediately or with a preceding period of cold temperature. His cultures were kept for a year and a half. Good germination was obtained for *V. nudum* and *V. opulus* in all conditions. Plantings of *V. alnifolium*, *V. cassinoides*, and *V. dentatum* were only successful when kept in the greenhouse from the beginning of the experiment. No greenhouse temperatures were given.

Barton (2, 3) reported that tree peony and lily seeds germinated readily

at a daily alternating temperature of 15° to 30° C. and a constant temperature of 20° C. but required a cold period to break the dormancy of the epicotyl.

In the following experiments an effort was made to work out in detail the germination and shoot development of various species of *Viburnum*.

MATERIAL AND METHODS

In the experiments described below work was done with the following species: *Viburnum acerifolium* L., *V. dentatum* L., *V. dilatatum* Thunb., *V. lentago* L., *V. nudum* L., *V. opulus* L., *V. prunifolium* L., *V. pubescens* Pursh., *V. rufidulum* Raf., and *V. scabrellum* (T. & G.) Chapm. The seeds of *V. acerifolium*, *V. dilatatum*, *V. opulus*, *V. prunifolium*, and *V. pubescens* were collected on Boyce Thompson Institute property. *V. dentatum* and *V. lentago* fruits were furnished by Thomas Lane, seedsman, Dresher, Pennsylvania. The fruits of *V. nudum*, *V. rufidulum*, and *V. scabrellum* were collected and sent through the courtesy of Teas Wholesale Nursery of Conroe, Texas.

The fruit of *Viburnum* is a one-seeded drupe with soft pulp and a thin stone. For the germination and seedling production tests the stones, referred to in this paper as seeds, for convenience, were freed from the pulp immediately. In a limited test on the effect of storage at room temperature and 5° C., seeds were stored in pulp as well as clean.

Experiments as conducted may be divided into germination tests where root production was recorded and seedling production in soil where shoot production was recorded. As all *Viburnum* species studied showed a similar trend in behavior, *V. acerifolium* will be described thoroughly as a type, while the other species will be discussed in a general way, except where deviations from the pattern of *V. acerifolium* are shown.

Constant and daily alternating temperatures were used. In the case of the latter, the cultures were left at the higher temperatures for 8 hours and at lower temperatures 16 hours each day.

EXPERIMENTAL RESULTS

GERMINATION

Cleaned seeds of all species were mixed with moist granulated peat moss and placed at constant temperatures of 1°, 5°, 10°, 15°, 20°, 25°, and 30°, C., and daily alternating temperatures of 10° to 30°, 15° to 30°, and 20° to 30° C. Different *Viburnum* species varied in germination rate and in range of effective temperatures. Of the temperatures tested those satisfactory for growing the first root of all species were constant 20° C. and 20° to 30° C. daily alternation. A daily alternation of 15° to 30° C. was slightly less effective and seeds germinated to a less extent and more slowly at 10° to 30° C. daily alternation. Germination of seeds of *V. aceri-*

folium, *V. rufidulum*, and *V. dentatum*, was spread over a long period (6 to 17 months) and took place only at a constant temperature of 20° C. and daily alternating temperatures of 10° to 30° C. and 20° to 30° C. For *V. prunifolium* and *V. dilatatum*, germination was completed in a much shorter period (seven to nine months). Germination of *V. lentago* was nearly completed after five months, and *V. opulus* after two months at the optimum temperatures. *V. lentago* and *V. opulus* also showed a broader range of effective temperatures than the forms mentioned above. Seeds germinated at 15° C. and 25° C., as well as at 20° C., 10° to 30° C., and 20° to 30° C. Seeds of *V. nudum* and *V. scabrellum* showed prompt and
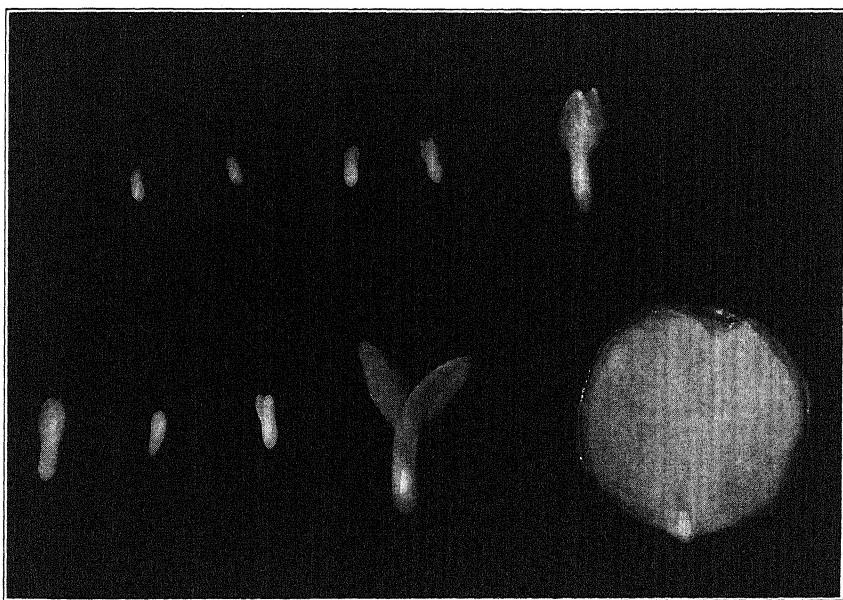
Figure 1. Embryos of *V. acerifolium*, excised after five months in peat at various temperatures. Left to right: (upper row) dry seed, 5°, 10°, 15°, 20° C.; (lower row) 25°, 30°, 10° to 30°, 20° to 30° C., cross section through seed.

complete root production. Germination for these two species took place through the entire temperature range from 5° to 30° C., including high alternating temperatures. Germination occurred during the first month and was practically completed at the end of the second month. These two species also failed to exhibit the epicotyl dormancy shown by the other forms studied as will be noted in data presented below. This was to be expected in view of the southern range of these forms.

The development of the embryos at various temperatures is shown in Figure 1, and indicates clearly the effective temperatures for germination

of *V. acerifolium.* The embryos were excised after five months in moist granulated peat moss at the various temperatures.

Removing the outer seed coat or treating seed coats with concentrated sulphuric acid for 15, 20, 25, and 30 minutes prior to placing in the germination medium had no effect on germination in most cases. Germination was decreased slightly by these treatments in a few instances. The germi-

TABLE I

Seedling Production of Viburnum Species, 1929 Crop, From Outside Plantings Made in November 1929 and Kept in Cold Frames Over Winter. Duplicate Samples of From 400 to 700 Seeds Each Used

Species	Per cent seedling production spring 1931		
	Open	Board-covered	Mulched
V. acerifolium A	0	17	17
V. acerifolium B	10	27	21
V. dentatum	12	57	31
V. dilatatum	9	36	28
V. lentago	7	27	27
V. opulus	20	47	45
V. prunifolium	8	45	55

TABLE II

Effect of Various Storage Conditions on Seedling Production of Viburnum Species in a Mulched Cold Frame. Seeds Planted in April 1930. Duplicate Lots of 100 Seeds Each Used for Each Test

Species	Per cent seedling production spring 1931					
	Storage					
	R. temp.			5° C. room		
	a	b	c	a	b	c
V. acerifolium	2	2	0	1	2	0
V. dentatum	17	17	5	22	30	17
V. dilatatum	19	18	5	24	26	10
V. lentago	27	22	3	37	49	16
V. opulus	37	24	13	62	45	31
V. prunifolium	27	22	7	27	21	7
V. rufidulum	27	22	12	31	24	0

a = Cleaned before storage.
b = Stored in pulp—cleaned before planting.
c = Stored in pulp—planted in pulp.

nation of seeds of *V. acerifolium,* which were stored in pulp at room temperature and cleaned before the acid treatment, was reduced from 59 per cent for untreated seeds to 15 per cent for a 20-minute and to 5 per cent for a 30-minute acid treatment. It is possible that the cleaning of dried fruits injured the seed coats so that the acid penetrated more easily.

SEEDLING PRODUCTION

Preliminary results. When seeds were planted in soil in flats in the fall of 1929 and wintered in various cold frame conditions, seedlings did not appear after the first winter but came up early in spring after the second winter. Table I shows these results. It was obvious that,for all species the

TABLE III

Effect of Planting Viburnum Seeds at Various Times During the Year and Wintering in Mulched or Board-Covered Cold Frame

Species	Storage	Per cent seedling production in spring 1937 from 1935 crop planted:											
		April 1, 1936		May 1, 1936		June 1, 1936		July 1, 1936		Aug. 15, 1936		Sept. 15 1936	
		B.C.	M.	B.C.	M.	B.C.	M.	B.C.	M.	B.C.	M.	B.C.	M.
V. acerifolium	R.T., cleaned	33	18	23	19	3	2	2	0	—	—	0	0
	R.T. in pulp*	21	13	9	7	1	1	1	0	—	—	0	0
	5° C., cleaned	40	28	55	40	17	18	15	3	—	—	0	0
	5° C. in pulp*	35	40	42	36	13	20	10	3	—	—	0	0
V. dentatum	R.T., cleaned	35	31	35	39	32	34	32	21	—	—	0	0
	R.T. in pulp*	30	30	23	21	22	23	18	14	—	—	0	0
	5° C., cleaned	31	31	32	21	30	32	26	19	—	—	0	0
	5° C. in pulp*	20	18	8	10	10	13	11	12	—	—	0	0
V. dilatatum	R.T., cleaned	79	—	69	—	60	—	34	—	—	—	0	—
	R.T. in pulp*	55	—	82	—	61	—	51	—	—	—	0	—
	5° C., cleaned	79	—	88	—	79	—	54	—	—	—	0	—
	5° C. in pulp*	81	—	72	—	77	—	77	—	—	—	0	—
V. prunifolium	R.T., cleaned	2	4	33	21	2	2	0	1	0	0	0	0
	R.T. in pulp*	9	6	17	23	5	1	0	1	0	0	0	0
	5° C., cleaned	10	7	26	33	0	2	1	3	0	0	0	0
	5° C. in pulp*	16	20	32	—	1	3	1	0	0	0	0	0
V. opulus	R.T., cleaned	57	—	69	—	88	—	46	—	56	—	0	0
	R.T. in pulp*	29	—	24†	—	54	—	66	—	62	—	0	0
	5° C., cleaned	60	—	64	—	76	—	87	—	1	—	0	—
	5° C. in pulp*	67	—	60	—	80	—	77	—	36	—	0	—

* Fruits were freed from pulp on the day before planting.
† Covered with moss.
R.T. = Room temperature.
B.C. = Board-covered frame.
M. = Mulched frame.

board-covered and mulched frames gave a higher percentage of seedling production than the open frame, where probably many seeds had been killed by freezing. The percentages in these tests were low, due to the fact that seedlings had already appeared and many had damped off when the frames were opened in March.

Table II gives the results of another preliminary test, where seeds of *Viburnum* species, stored at various conditions for five months, were planted in a cold frame in April 1930. Seedling production occurred the

following spring. Fairly good stands of seedlings were obtained for all species except *acerifolium*. Comparing the data of Tables I and II, it will be observed that seeds planted in November 1929 or April 1930 produced seedlings at the same time (spring 1931). It therefore becomes obvious that only one cold period is essential for the development of seedlings of most *Viburnum* species but it is also shown that the cold period must be preceded by a warm period in order to be effective.

High temperature. Experiments were conducted to work out the most effective length of high temperature for seedling production. Seeds of *V.*
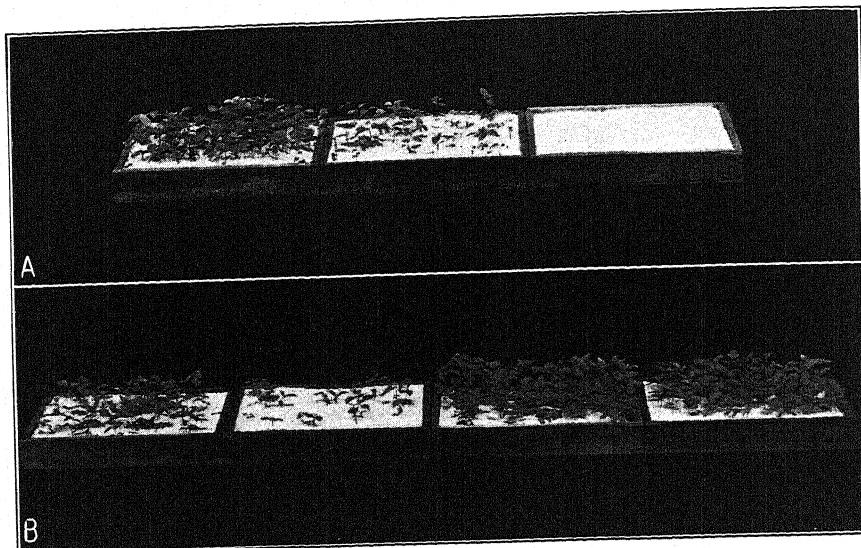
FIGURE 2. (A) *V. acerifolium.* The effect of various planting times during summer on seedling production the following spring. Left to right: Planted April 1st, June 1st, September 15th, 1936. (B) The effect of storage on seedling production of *V. acerifolium* planted May 1, 1936. Left to right: Room temperature cleaned, in pulp; 5° C. cleaned, in pulp.

acerifolium, *V. dentatum*, *V. dilatatum*, *V. prunifolium*, and *V. opulus* were planted in flats every month during the summer of 1936, starting with April 1. The flats were wintered in board-covered and mulched frames and in a greenhouse of 21° C. Table III shows that seeds of *V. acerifolium* produced up to 40 and 55 per cent seedlings after one winter when planted in April or May. Seedling production decreased rapidly for plantings in June and later, with none at all for the plantings in September (Fig. 2 A).

The effective length of exposure to warm temperature followed by a cold period varied for the species of *Viburnum* and confirmed the results from germination tests in moist peat moss at controlled temperatures.

The response of *V. prunifolium* to high temperature was somewhat similar to that of *V. acerifolium*, with 33 per cent seedling production for the May planting, and scarcely any for the ones later than May. The July plantings of seeds of *V. dilatatum* still gave 77 per cent and of *V. dentatum* 32 per cent seedling production. August plantings of *V. opulus* showed a percentage of 62. The flats kept at 21° C. for the duration of the experiment showed occasional weak seedlings.

TABLE IV

EFFECT OF PRE-TREATMENT OF GERMINATED SEEDS OF VIBURNUM ACERIFOLIUM AT VARIOUS LOW TEMPERATURES ON SHOOT PRODUCTION IN THE GREENHOUSE. CROP 1932

Pre-treatment		Per cent shoot production after months in the greenhouse (about 21° C.)					
Temp. ° C.	Months	0.5	1	1.5	2	2.5	3
3	1	0	0	0	5	6	9(7)*
	1.5	0	3	4	7	9(5)	
	2	1	3	4	4		
	2.5	3	4	7			
	3	1	2	4			
	4	16					
5	1	0	1	4	11	12	
	1.5	0	12	35	42	46	
	2	0	29	49			
	2.5	13	61	61			
	3	6	49	51			
	4	51					
10	1	0	9	23	30	35(20)	
	1.5	0	9	27	33(7)		
	2	1	13	31	31(2)		
	2.5	3	25	36	40		
	3	34	60	67(6)			
	4	77	81(2)				
15	1	0	2	9	13	19	
	1.5	0	7	13	13		
	2	5	9	17	19		
	2.5	4	7	8			
	3	7	13				
	4	13					
None		0	0	0	1	1	2(2)

* Numbers in parentheses indicate seedlings above soil but with seed coats attached.

Low temperature. To determine the optimum length of time at low temperatures and also the optimum low temperature needed to overcome the epicotyl dormancy typical for most species of *Viburnum*, seeds of *V. acerifolium*, *V. dentatum*, *V. dilatatum*, *V. prunifolium*, and *V. opulus*, pre-germinated in peat at 20° to 30° C. daily alternation, were planted in pots and kept in rooms of 3°, 5°, 10°, and 15° C., as well as in a greenhouse of 21° C. Series of pots were transferred from the various rooms to the

greenhouse every half month. The optimum cold treatment for *V. aceri-folium* was two and one-half months at 5° C., with 61 per cent shoot development and four months at 10° C. with 81 per cent (Table IV and Fig. 3).
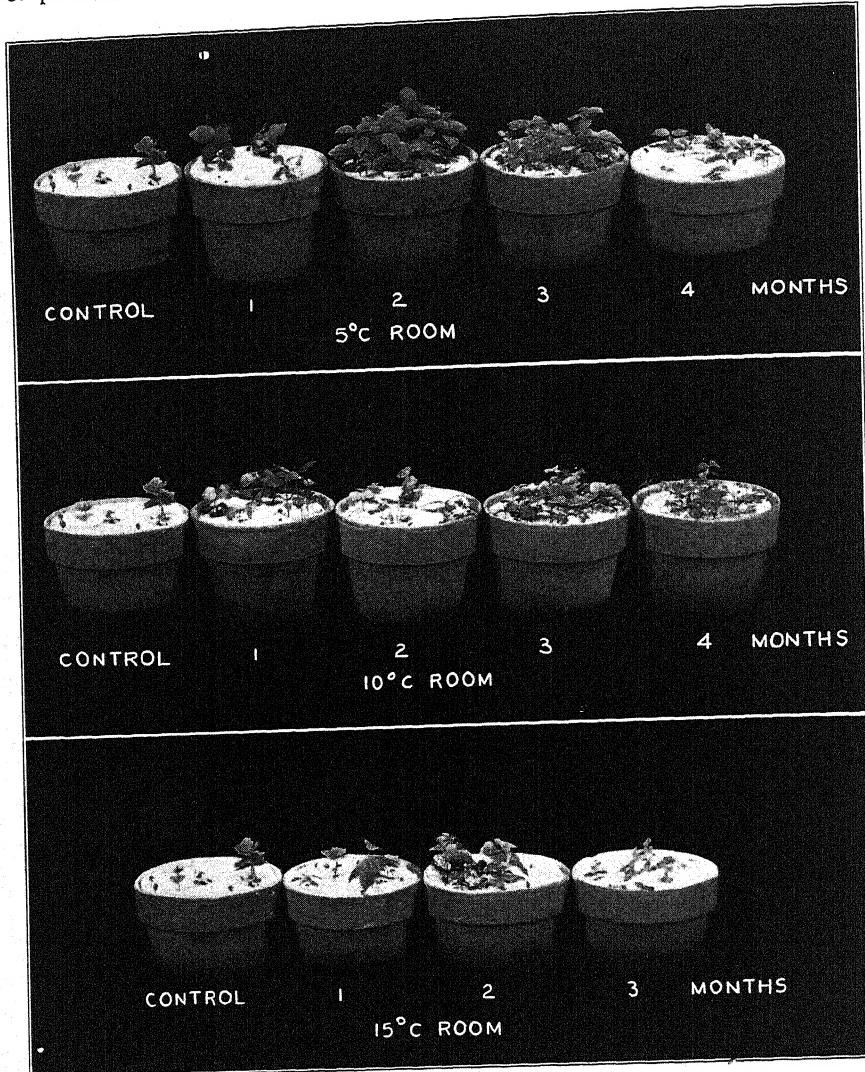
FIGURE 3. *V. acerifolium.* The effect of low temperature pre-treatment of germinated seeds on shoot development at 21° C.

Shoot development was very poor for the cultures at 3° and 15° C. and practically failed in the greenhouse. Figure 3 shows excellent shoot development after two and three months at 5° C., after three and four

months at 10° C., and poor results from 15° C. Removing the outer seed coat was without effect or in some cases slightly injurious to shoot development of *V. acerifolium*.

Comparable treatments for breaking epicotyl dormancy were conducted using germinated seeds of *V. dentatum*, *V. dilatatum*, *V. opulus*, and *V. prunifolium*. Their behavior was similar to that of *V. acerifolium*, except that the effective range of low temperatures and the length of the exposure time for optimum results varied. *V. acerifolium* showed greatest epicotyl dormancy. Only 5° and 10° C. gave good results (Fig. 4 A). *V. dentatum* gave 53 per cent shoot production after only one-half month at 5° C., and 69 per cent after one-half month at 10° C. Up to 85 per cent was obtained after two and one-half months at 10° C. Three degrees C., as well as 15° C., was inferior, but the entire range of low temperatures used was effective. Seeds of *V. dentatum* would produce up to 41 per cent seedlings in the greenhouse without any cold pre-treatment, but such seedlings were always poorly developed.

V. dilatatum (Fig. 4 B) showed 94 and 91 per cent seedling production after three months at 5° C., and after four months at 10° C. Three degrees C. was less favorable. Fifteen degrees C. gave very poor results; the greenhouse of 21° C. none at all.

V. opulus and *V. prunifolium* (Fig. 4 C) were less dormant than the species previously described. While 5° and 10° C. were slightly superior, good shoot development was also obtained from 3° and 15° C. Seedlings up to 19 per cent for *V. opulus* and up to 31 per cent for *V. prunifolium* occurred in the greenhouse of 21° C. without low temperature pre-treatment but here again seedlings were poorly developed.

V. lentago, *V. pubescens*, and *V. rufidulum* responded in the same way as the dormant types already discussed.

The two southern species, *V. nudum* and *V. scabrellum*, did not show any epicotyl dormancy. When seeds were planted in flats and kept at a high temperature seedlings came up in one to one and one-half months. When seeds were planted in flats in greenhouses of various temperatures seedlings grew somewhat more slowly in the cooler houses. At temperatures above 21° C. and when flats were alternated between 18° and 26° C. seedlings damped off easily.

The high percentages obtained throughout the epicotyl dormancy tests were due to the fact that only germinated seeds were used.

Effect of storage. In the preliminary tests described in Table II and for the flat plantings that were made every month throughout the summer (Table III), seeds that had been stored cleaned and in pulp at room temperature and at 5° C. were used. The seeds stored in pulp were cleaned before planting. Table III shows that storage at room temperature was inferior to storage at 5° C. for *V. acerifolium* but this effect was not evident

for the other species. There was some indication that seeds stored in pulp at room temperature deteriorated more rapidly than those cleaned before storage. This effect also was most obvious for *V. acerifolium*. Plantings in May (Fig. 2 B) gave 23 per cent seedling production for seeds
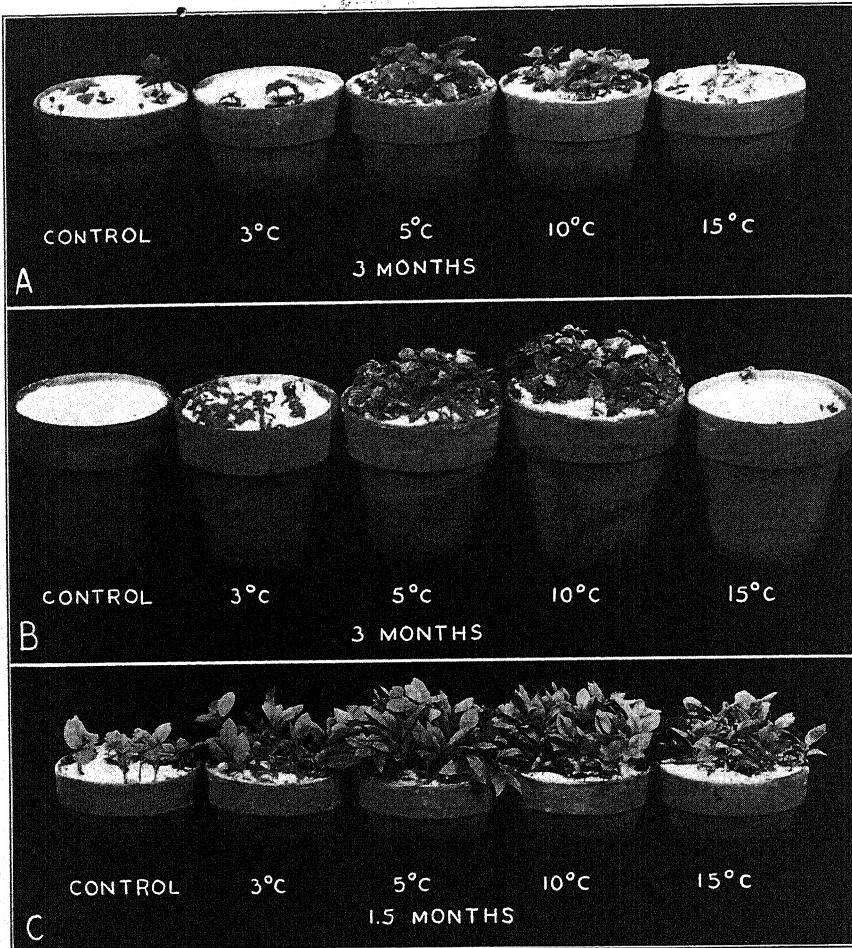
FIGURE 4. The effect of low temperature on shoot development of *Viburnum* species. (A) *V. acerifolium*. (B) *V. dilatatum*. (C) *V. prunifolium*.

stored cleaned, 9 per cent when kept in pulp, both stored at room temperature. Seedling production of seeds stored cleaned at 5° C. was 55 per cent, kept in pulp at 5° C. 42 per cent. These effects of storage were incidental to the work on seedling production of the genus *Viburnum*. Whether lower seedling production from seeds stored in pulp at room temperature

was actually a loss in vitality is a question. It could easily have resulted from injury of the seed coat during the process of cleaning the dry pulp from the seeds. These fruits had to be rubbed for a long time before the hardened pulp was removed. In the case of fruits stored at 5° C., except *V. dentatum* the pulp of which was dry when the seeds were received, the pulp would remain moist and could easily be removed. This difficulty in cleaning dried pulp from seeds might account for the apparent loss of vitality of *V. dentatum* stored in dried pulp at 5° C. (Table III).

SUMMARY

Viburnum species, except southern forms, required constant temperature of 20° C. or a daily alternating temperature of 20° to 30° C. for root production (germination) followed by a low temperature pre-treatment for shoot production (seedling production). The rate of germination and the effective temperature range varied for different *Viburnum* species. *V. acerifolium*, *V. dentatum*, and *V. rufidulum* germinated only within a narrow temperature range (20° C. constant, and 10° to 30° C. and 20° to 30° C. daily alternations) and required from 12 to 17 months for completion of germination. *V. dilatatum* and *V. prunifolium* responded to the same temperatures but required a shorter time for completion of germination (six to eight months). *V. lentago*, *V. nudum*, *V. opulus*, and *V. scabrellum* not only germinated more quickly but were less specific in their temperature requirements.

Seeds of the forms studied, except *V. nudum* and *V. scabrellum*, planted in flats in the fall and placed in open, mulched, or board-covered cold frames, produced seedlings only after the second winter. Similarly treated spring plantings, however, gave a good seedling crop after one winter.

Seeds of various *Viburnum* species, pre-germinated in moist peat moss at 20° to 30° C. daily alternation, were planted in pots and kept at various low temperatures and in the greenhouse of 21° C. Results of these tests showed the efficacy of low temperature treatment of germinated seeds, since prompt and complete appearance of healthy shoots followed the transfer of pots from low temperatures to a greenhouse at 21° C. Control pots left in the greenhouse for the entire period showed occasional weak shoot production.

Storage for seven months at 5° C. was more favorable for the retention of vitality of *V. acerifolium* seeds than storage at room temperature especially if the seeds were stored in pulp.

Failure of the shoot to emerge and grow at high temperatures is thought to be due to mechanical restriction and partial dormancy.

Seeds of *V. nudum* and *V. scabrellum* offer no problem in seedling production. However, *V. acerifolium*, *V. dentatum*, *V. dilatatum*, *V. lentago*,

V. opulus, *V. prunifolium*, and *V. rufidulum* must have a warm period for germination and root development followed by a cold period to force epicotyl development. These conditions are easily obtained on a commercial scale in regions with the general temperature of Yonkers, New York, by spring or early summer plantings outside. Seedlings will appear after one winter.

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SOME FACTORS AFFECTING GERMINATION AND GROWTH OF GENTIAN

JOHANNA GIERSBACH

INTRODUCTION

The growth of gentian plants from seeds has interested garden lovers, nurserymen, and scientists for many years. The problems encountered concern both germination and the further development of the seedlings. Although numerous reports of observations on or experiments with this genus have appeared in the literature from time to time, they have been short, and in no case has an investigation been consistently carried through the stages from germination to flowering of gentian plants. The effect of low temperatures and light on germination have been studied. Kinzel (8, 9, 10) emphasized the effect of light and freezing on germination. According to him, seeds which had been frozen dry or in the seed bed germinated in warm temperature. He mentioned *Gentiana acaulis* as one of the hardest to germinate. Doerfel (5) found one or more periods at 0° or 5° C. helpful. Norton (16) reported that best seedling production was obtained when fresh seeds were planted in the fall. However, seeds could be stored over winter in a cool place and keep their vitality. Nearing (13) sowed the seeds in November and kept them outside in a protected place.

According to Abbey (1) a constant supply of moisture is important. In places where this supply was lacking he found fringed gentians growing with low grasses. Bailey and Bailey say in their "Hortus" (3, p. 272): "Gentians require good drainage with plenty of moisture." Numerous other short articles have been published emphasizing the importance of the moisture relation in gentian culture.

The best type of soil for gentians has been the subject of several experiments. Norton (16) suggested filling the pots with one inch of gravel, two inches of sand, two inches of good garden soil baked to destroy weed seeds, and two inches of baked garden soil mixed with pulverized moss. Hedden (7) sifted sphagnum moss and mixed it with soil. Millard (12) suggested leaf mold without lime and rich in iron. Amsler (2) found that lime and acidity were incidental as long as good drainage was provided. Matschat (11) used sifted soil from places where gentians were growing, thinking a possible symbiotic relationship with soil organisms helpful to the development of gentian plants. Other workers have also thought of the latter possibility. Costantin and Magrou (4) found fungal associations for *Gentiana cihata* and *G. bararica*. Weiss (17) did not observe any mycorrhizal fungus in the seed coat of several gentian species, including *Gentiana acaulis*, nor did he find it in the roots of gentians. He mentioned the ab-

sence of root hairs. He believed that while roots of gentians in their natural habitat frequently contained endotrophic fungi, the latter were not needed for the development of the plant. Weiss planted seeds in boxes and wintered them outside with successful germination in spring. While Neumann (14) discovered a mycorrhizal fungus in the roots of some of the gentians, she could not find any in the roots of *G. acaulis*.

The present paper deals with some of the factors affecting germination, seedling production, and seedling development of three species of gentian. A preliminary report of storage effects on seeds of the fringed gentian is also given.

MATERIAL AND METHODS

The seeds of *Gentiana crinita* Froel. were obtained through the courtesy of Dr. G. F. Norton, Norwich, New York. Those of *Gentiana andrewsii* Griseb. were partly collected by Mr. Frederick M. Holbrook, White Plains, New York, and partly collected at the Boyce Thompson Arboretum. The seeds of *Gentiana acaulis* L. came from Hilchenbach, Westfalen, Germany.

The germination of seeds was tested on moist filter paper in petri dishes in electrically-controlled ovens and at room temperature. Tests for seedling production were made with various kinds of soil in the greenhouse with and without pre-treatment in cold rooms.

RESULTS

GENTIANA CRINITA

Germination. Seeds were placed on moist filter paper in petri dishes at a daily alternating temperature of 15° to 30° C., with 8 hours at 30° and 16 hours at 15° C., with and without preceding periods of various lengths at constant temperatures of −10°, 1°, 5°, and 10° C. and a daily alternation of −10° to 5° C. Samples were removed from the low temperature and placed at 15° to 30° C. daily alternation every month over a period of four months. According to the results shown in Table I, 1° C. was best for pre-treatment, and one month was as effective as longer periods. Germination at 15° to 30° C. daily alternation when preceded by a period at 10° C., the data of which are not given in this table, was much inferior to either 1° or 5° C. When freezing or freezing and thawing preceded 15° to 30° C. daily alternation, germination was very poor. This confirms Doerfel's findings (5) that not freezing but low temperatures were essential for after-ripening certain gentian seeds. Daily alternation of 15° to 30° C. without previous low temperature treatment gave only 2 per cent germination.

Seedling production. Seeds were planted in pots on top of a soil mixture of sand, leaf mold, sod soil, and clay in equal parts, watered from below,

and kept in a greenhouse of 21° C. with and without preceding periods of two, three, and four months at constant temperatures of − 10° C., 3° C., and 5° C. and a weekly alternation of − 10° to 5° C. One inch of gravel was placed in the bottom of each pot for good drainage. In the greenhouse the pots were covered with a glass plate to prevent the surface of the soil from drying out. When the pots were left for two months at 3° or 5° C. good seedling production resulted when they were transferred to the greenhouse (Table I and Fig. 1, top row). If pre-treatment of seeds planted in soil was continued for longer than two months, 3° C. was preferable to 5° C. since deterioration of the seeds took place after three or four months at the latter temperature with a subsequent falling-off in

TABLE I

EFFECT OF LOW TEMPERATURE PRE-TREATMENT ON GERMINATION OF GENTIANA CRINITA AT 15° TO 30° C. DAILY ALTERNATION AND ON SEEDLING PRODUCTION IN THE GREENHOUSE

Germ. temp.	Pre-treatment, temp. ° C.	% germination at 15° to 30° C. or seedling production in the greenhouse after months at low temperature				
		None	1	2	3	4
15° to 30° C. daily alternation	− 10		3	3	4	1
	− 10 to 5 daily alternation		5	15	17	11
	1		30	36	29	38
	5		23	19	15	24
	None	2	—	—	—	—
Greenhouse	− 10		—	6	4	3
	− 10 to 5 weekly alternation		—	8	8	1
	3		—	32	32	27
	5		—	31	18	19
	None	2	—	—	—	—

seedling production. Freezing and alternating freezing and thawing were unfavorable. One month at 10° C. had no effect on seedling production at 21° C., while fair results were obtained after two months at 10° C. The data of these results are not given in this paper.

Seedling development. One of the principal problems encountered in raising gentian from seed is that of successfully growing the young seedlings. Pure sand as well as a number of different soil mixtures were tested here. Some seedlings failed to become established regardless of the soil mixture, so that after a time there was a decrease in the number of plants. This decrease was much more marked in the unfavorable soils (Fig. 1, bottom row). In mixture A containing sand, leaf mold, and clay, the number of seedlings was reduced from 43 to 37 per cent in three and one-half months. In mixture B composed of sand, sod soil, and clay, the percentage dropped from 51 to 11 in the same time. In a mixture of sand, sod

soil, and leaf mold (C), seedlings were reduced in number from 64 to 47 per cent. For soil made up of sand, sod soil, leaf mold, and clay in equal parts (D), the number of seedlings decreased from 33 to 29 per cent. Leaf mold was an essential part of a soil mixture good for the growth of fringed gentians, perhaps because of its water-holding capacity. Seedlings developed most rapidly and with a healthy dark green color when moss was growing around them. On all soils containing leaf mold, moss also grew best. In pure sand the surface dried out too easily to be effective.
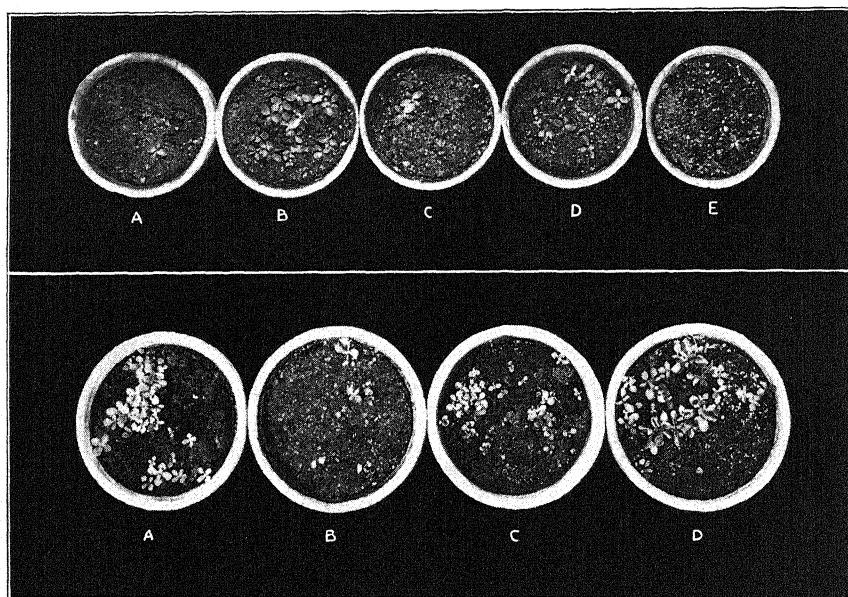
FIGURE 1. *Gentiana crinita.* Top row: Effect of low temperature pre-treatment for 2 months on seedling production in the greenhouse. (A) — 10° C.; (B) 3° C.; (C) 5° C.; (D) — 10° to 5° C. weekly alternation; (E) no pre-treatment. Bottom row: The effect of soil mixture on seedling development. (A) Sand, leaf mold, and clay; (B) sand, sod soil, and clay; (C) sand, leaf mold, and sod soil; (D) sand, sod soil, leaf mold, and clay.

After the development of four to six leaves, as shown in Figure 1, bottom row, seedlings could be planted in individual pots in a richer soil, during which process care was taken not to disturb the roots unnecessarily. The best time for doing this was in May. This gave the seedlings a chance to grow into strong plants before fall. With the beginning of cold weather the leaves died back and the seedlings remained dormant until the following spring.

In order to determine whether a cold period was needed for normal growth and flower bud development during the second summer, seedling

plants were placed in greenhouses of 7° C. and of 21° C. Every month from December 1st, 1936 to March 1st, 1937, plants were transferred from 7° C. to 21° C. Seedlings were measured each month from February to June. The lengths are reported in centimeters in Table II. Seedlings with two or three months of cold grew more rapidly and taller than the ones with one or four months at 7° C. However, they bloomed at the same time (May 15th). Most of the seedlings kept at 21° C. for the entire duration of the experiment died. The few surviving ones grew normally at first, but later developed slowly and acquired a stunted appearance which was still evident on June 15th. At that time the plants with two and three months of cold treatment were well developed and full of flower buds. Those with two months of cold were in full bloom. Those given one

TABLE II

EFFECT OF A COLD PERIOD ON SEEDLING DEVELOPMENT OF GENTIANA CRINITA. SEEDLINGS WERE BROUGHT INTO UNHEATED GREENHOUSE ON APRIL 15, 1937. SAMPLES OF 8 SEEDLINGS EACH WERE USED

Months in green-house at 7° C.	Average size of seedlings in cm.														
	7° C.					21° C.					21° C. +artificial light				
	Date of measuring														
	Feb. 1	Mar. 1	Apr. 1	May 15	June 15	Feb. 1	Mar. 1	Apr. 1	May 15	June 15	Feb. 1	Mar. 1	Apr. 1	May 15	June 15
Constant	1	1.6	3.9	10.6	16.6										
1						4.1	5.2	8.1	16.4*	25*	3.4	5.1	9.7	21.3*	27.2*
2						4	5.8	12.6	30.1*	35*	4.9	9.7	20.9	38.4*	42.7*
3							3.6	8.3	18.6*	30.8*					
4									5.4	13.9*	23.1*				
None						2.5	3.3	5	11.5	21.5*	3.8	6.9	10.5	21.5*	31.5*

* Blooming.

and four months of cold were slightly retarded. The seedlings kept at 7° C. all winter developed like normal plants outside to be ready to bloom in the fall. Figure 2 shows the seedlings with various treatments as they appeared in February, April, and June. Here temperature effects on development are clearly shown.

Artificial light supplied by 500-watt Mazda lamps for four hours, in the course of each 24 hours, chiefly at night as a supplement to daylight, combined with 21° C., increased the height of the plants regardless of their temperature pre-treatment (Table II). The effect of the extra light was especially notable in the case of plants which received no cold treatment, bringing them into flower in May instead of June. However, the mortality of these seedlings was high.

Storage. Seeds of the 1935 crop were stored open and sealed in air and in a partial vacuum at room temperature and at 5° C. Germination of
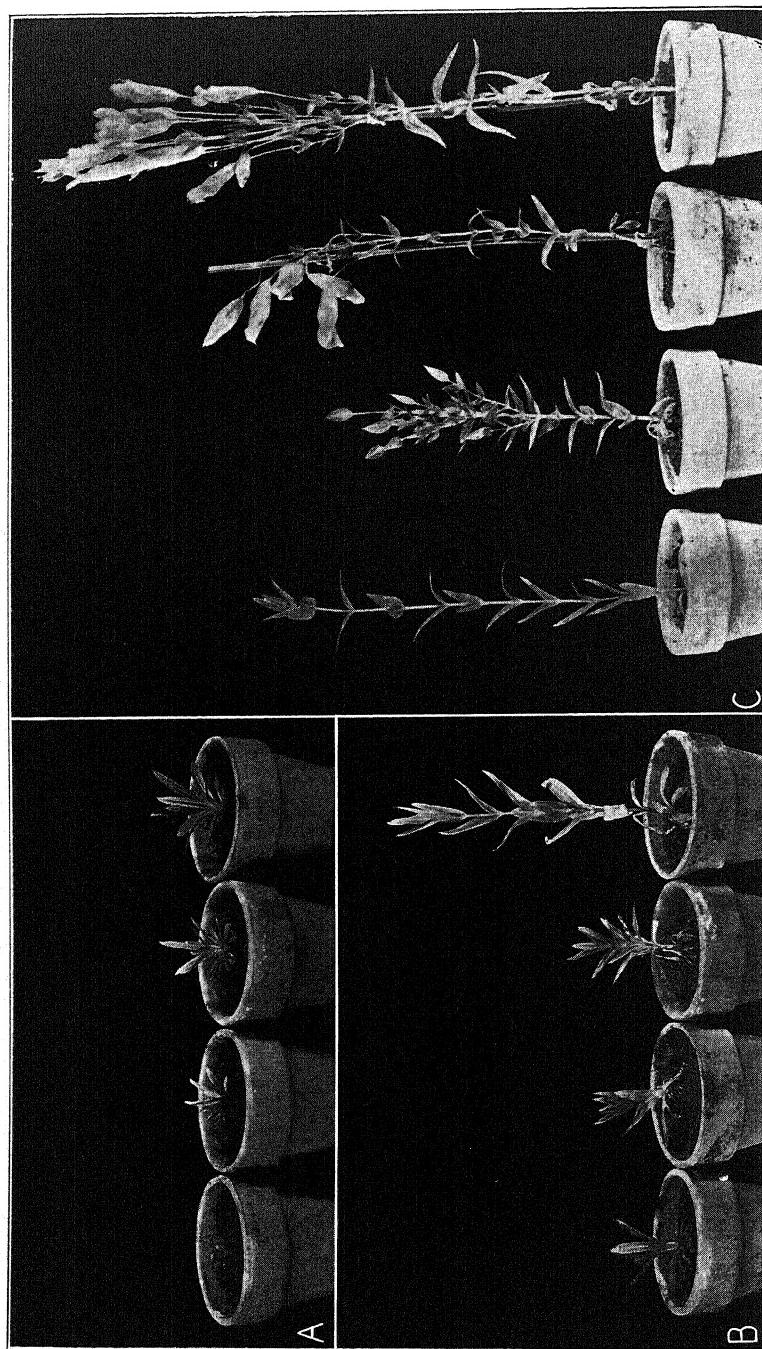
FIGURE 2. *Gentiana crinita.* Seedling development. Photographed (A) in February; (B) in April; (C) in June. Pots treated in greenhouses at various temperatures. Left to right in each row: At 7°C. until warm weather; at 21°C.; 1 month at 7°C. then 21°C.; 2 months at 7°C. then 21°C.

the stored seeds was tested on moist filter paper at 15° to 30° C. daily alternation with a preceding period of two months at 1° C. Table III shows that germination decreased to one per cent for seeds stored open at 5° C. for six months while those stored open at room temperature gave 22 per cent germination. There was some decrease of germination for all other storage conditions. Practically no germination occurred at 15° to 30° C. daily alternation without preceding low temperature from seeds of any storage condition. After one year of open storage both at room tempera-

TABLE III

EFFECT OF VARIOUS STORAGE CONDITIONS ON GERMINATION OF GENTIANA CRINITA AT 15° TO 30° C. DAILY ALTERNATION AND ON SEEDLING PRODUCTION IN THE GREENHOUSE

| Germ. temp. | Storage | | % germination at 15° to 30° C. or seedling production in the greenhouse after months of storage | | | | | |
| | | | None | | 6 | | 12 | |
	Temp.	Condition	a	b	a	b	a	b
15° to 30° C. daily alternation	Room	Open			o	22	o	o
		Sealed air			o	18	1	7
		Vacuum			o	28	1	32
	5° C.	Open			o	1	o	o
		Sealed air			o	27	1	31
		Vacuum			o	18	o	29
	Fresh seeds		2	36				
Greenhouse	Room	Open			o	17	1	1
		Sealed air			o	23	2	8
		Vacuum			o	28	14	56
	5° C.	Open			o	2	1	2
		Sealed air			1	10	10	34
		Vacuum			1	26	3	42
	Fresh seeds		2	31				

a = Without low temperature pre-treatment.
b = 15° to 30° C. daily alternation after two months at 1° C., greenhouse after two months at 3° C.

ture and 5° C. seeds had lost their vitality. Germination of seeds stored sealed in air at room temperature had dropped to 7 per cent, while seeds stored in the same condition at 5° C. showed only a slight decrease in germination compared with the data from fresh seeds. Sealing seeds in a vacuum was especially effective when stored at room temperature. The seeds under such conditions maintained complete germinating power for 12 months as against an 80 per cent loss of vitality for seeds sealed in air at the same temperature. When the storage temperature was favorable for the retention of vitality, the vacuum had no effect up to 12 months of

storage. Results of the soil plantings confirmed those of the filter paper tests. It will be noted, however (Table III), in the case of soil tests that an apparent after-ripening in dry storage has taken place after 12 months, since up to 14 per cent seedling production was obtained without low temperature pre-treatment. This effect was not shown in the filter paper cultures, thus indicating that soil is a better medium than filter paper for the germination of untreated seeds. For pre-treated seeds, however, the

TABLE IV

EFFECT OF VARIOUS STORAGE CONDITIONS ON GERMINATION OF GENTIANA CRINITA AT 15° TO 30° C. DAILY ALTERNATION AND ON SEEDLING PRODUCTION IN THE GREENHOUSE. CROP 1936

Germ. temp.	Storage		% germination at 15° to 30° C., or seedling production in the greenhouse after months of storage									
			0		1		2		3		4	
	Temp.	Condition	a	b	a	b	a	b	a	b	a	b
15° to 30° C. daily alternation	Room	Open			0	68	0	82	1	61	2	65
		Sealed Air			0	68	3	82	2	66	0	50
		Sealed Vac.			0	66	0	74	1	63	0	53
		Sealed N			0	67	0	69	0	74	0	62
	5° C.	Open			0	61	0	62	0	62	0	40
		Sealed Air			0	67	0	56	1	69	0	36
		Sealed Vac.			1	81	0	76	0	66	0	43
		Sealed N			0	77	0	64	1	65	0	58
	Fresh seeds		0	79								
Greenhouse	Room	Open			8	55	16	81	22	51	8	51
		Sealed Air			10	57	14	74	16	55	2	35
		Sealed Vac.			16	73	11	86	23	61	8	39
		Sealed N			29	63	9	82	40	68	5	64
	5° C.	Open			15	69	19	78	30	52	2	47
		Sealed Air			20	62	10	78	20	68	2	52
		Sealed Vac.			11	41	11	78	9	46	8	19
		Sealed N			6	15	15	87	8	16	6	33
	Fresh seeds		15	67								

a = Without low temperature pre-treatment.
b = 15° to 30° C. daily alternation after two months at 1° C., greenhouse after two months at 3° C.

medium is not so important. Table IV shows the results of storage tests of the 1936 crop where seeds were stored open and sealed with air, with nitrogen, and in a partial vacuum. Germination was tested as for seeds of the 1935 crop, except that 5° C. was used in addition to 1° C. for pre-treatment for the oven tests. Since 5° C. proved slightly inferior to 1° C. as an after-ripening temperature, these data are not included in the table. It will be noted that fresh seeds of this crop gave higher germina-

tion percentages than those of the 1935 crop (79 and 36 per cent respectively). Vitality tests of stored seeds of the 1936 crop made after one, two, three, and four months indicated that there was practically no loss in germinating power under any of the conditions tried with the possible exception of a slight loss under some conditions at the four-month period. Neither fresh nor stored seeds germinated without pre-treatment at low temperature.

Again, the soil plantings confirmed the results of the filter paper tests, with the exception of the germination of seeds without cold pre-treatment. The superiority of soil over filter paper as a medium is shown in this less dormant seed crop where fresh as well as stored seeds produced some seedlings without low temperature pre-treatment (Table IV).

GENTIANA ANDREWSII

Germination. Nichols (15) reported that seeds of *Gentiana andrewsii* germinated up to 49 per cent when exposed to 83 days of cold.

TABLE V

EFFECT OF LOW TEMPERATURE ON GERMINATION OF GENTIANA ANDREWSII

Pre-treatment (° C.)	% germination on moist filter paper after months at low temperature							
	1		2		3		4	
	a	b	a	b	a	b	a	b
−10 to 5*	0	0	9	53	28	34	48	64
1 to 10*	16	8	55	65	71	88	79	81
1	24	26	58	69	63	53	71	75
5	9	8	19	57	16	40	48	57
10	2	3	24	38	34	50	26	45
15	0	0	0	16	0	2	1	0

a = 15° to 30° C. daily alternation.
b = Room temperature in light.
* Daily alternation.

In the present paper experiments are described where seeds of this species were placed on moist filter paper in petri dishes at 15° to 30° C. daily alternation and in light at room temperature. Seeds were also kept at constant temperatures of 1°, 5°, 10°, and 15° C. and at daily alternating temperatures of −10° to 5° C. and 1° to 10° C. Samples were transferred monthly from these low temperatures to 15° to 30° C. and to room temperature in the light for a period of four months. Slightly higher percentages were obtained in practically all cases where room temperature coupled with light was used for germination (Table V). This was a favorable light effect since similar seeds kept in the dark at room temperature showed less germination. Seeds germinated at room temperature in light with 26 per cent after one month and with 75 per cent after four months

at 1° C. One month at 5° and 10° C. gave very poor germination, but 57 and 50 per cent occurred when two months at 5° C. or three months at 10° C. preceded high temperature. Freezing (-10° C.) to 5° C. was as effective as 5° C. and 10° C. when applied for four months. Such treatment resulted in 48 per cent germination at 15° to 30° C. daily alternation and 64 per cent in light at room temperature. A daily alternation of 1° to 10° C. resulted in 88 per cent germination when the cultures were transferred to light at room temperature. Pre-treatment at 15° C. proved

TABLE VI

EFFECT OF VARIOUS LOW TEMPERATURES AND OF VARIOUS SOILS ON SEEDLING PRODUCTION OF GENTIANA ANDREWSII IN THE GREENHOUSE

Pre-treatment		% seedling production after two weeks in the greenhouse		
Low temp., ° C.	Months	Soil mixture		
		a	b	c
3	1	11	41	58
	2	37	42	62
	3	29	15	22
	4	6	15	40
5	1	57	81	71
	2	59	36	45
	3	25	16	50
	4	25	17	38
10	1	41	51	68
	2	53	61	59
	3	40	49	43
	4	33	31	40
None		58*	60*	56*

a = Sand, leaf mold, and clay in equal parts.
b = Sand, sod soil, and clay in equal parts.
c = Sand, leaf mold, clay, and sod soil in equal parts.
* Majority of seedlings damped off as soon as they appeared above ground.

ineffective. Germination failed completely at 15° to 30° C. daily alternation and was very poor in light at room temperature without low temperature pre-treatment.

Gassner (6) reported that seeds of some gentians that were light-sensitive could be stimulated by nitrates. As seeds of *Gentiana andrewsii* responded to light, seed samples of this species treated with 0.05 M and 0.005 M solutions of KNO_3 were compared with samples treated with tap and distilled water. Seeds were placed on filter paper soaked in various solutions and kept at 15° to 30° C. daily alternation and at room temperature in light and dark. In the latter case black petri dishes were used. Treated samples were also placed at 1°, 5°, and 10° C. for one and two

months and transferred from these to 15° to 30° C. daily alternation for germination. Results of these tests indicated that KNO_3 in the concentrations used was not effective in replacing either the need for light or the low temperature required for germination. Soaking seeds in these solutions for 24 hours before planting in soil was also ineffective.

Seedling production. Seeds were planted in three-inch pots on top of three different soil mixtures, containing (A) sand, leaf mold, and clay, (B) sand, sod soil, and clay, and (C) sand, leaf mold, clay, and sod soil in equal parts. The pots were kept in a greenhouse of 21° C. or in rooms at 3°, 5°, and 10° C. Pots were transferred from the cold temperatures to 21° C. after one, two, three, and four months. One month at 5° C. resulted in 57, 81, and 71 per cent seedling production in the greenhouse (Table VI). A period longer than one month at 5° C. reduced seedling production considerably. This unfavorable effect was most marked for soil B which contained no leaf mold. Seeds tended to decay when left too long in the cold. Samples with one month at 3° and 10° C. produced seedlings as high as 58 per cent for 3° C. and 68 per cent for 10° C. Seeds in pots at 21° C. without preceding low temperature germinated up to 60 per cent. However, the majority of these seedlings damped off as soon as they came up, whereas seeds pre-treated in a cold room produced healthy plants.

Seedling development. The effect of temperature on the development of the seedlings was also studied for *Gentiana andrewsii*. Tests similar to those with *Gentiana crinita* were carried out. Unlike the latter, *G. andrewsii* seedlings do not die back in the fall since the plants are perennials. Cold treatment effects on seedling development during the second winter gave results similar to those for fringed gentian as regards flower bud development. However, the growth of the entire plant was different. Previous to April, plants with some cold treatment grew faster than the ones kept at 21° C. constantly. After April, growth of cold-treated plants became more and more stunted and only terminal flower clusters formed. The plants with a complete winter kept on growing like normal plants outdoors.

<center>GENTIANA ACAULIS</center>

Germination. Seeds were placed on moist filter paper at 3°, 5°, and 10° for one, two, and three months, after which period they were transferred to 15° to 30° C. daily alternation. Samples were also kept at 15° to 30° C. without low temperature pre-treatment. Table VII shows that one month at 1°, 5°, or 10° C. was not sufficient to bring about germination. Germination occurred after two and three months at 1° C. (36 and 16 per cent). Five degrees C. proved inferior to 1° C., and 10° C. was without effect. No germination was obtained at 15° to 30° C. without cold pre-treatment.

Seedling production. Table VII gives the data obtained from plantings in pots on top of soil. The pots were kept in a greenhouse at 21° C.

and in cold rooms at 3°, 5°, and 10° C. and transferred to 21° C. for seed-
ling production. One month of cold gave very poor seedling production,
41 per cent was obtained after two months, and 39 per cent after three
months at 3° C.; two and three months at 5° C. gave only 23 and 17 per
cent; and again 10° C. proved ineffective. No seedlings occurred in pots
without cold treatment. It was found that for good growth, the seedlings
of *Gentiana acaulis* should not be kept as wet as the fringed and closed
gentians.

TABLE VII

EFFECT OF LOW TEMPERATURE ON GERMINATION AND ON SEEDLING
PRODUCTION OF *GENTIANA ACAULIS*

Germ. temp.	Pre-treatment, ° C.	% germination at 15° to 30° C. or seedling production in the greenhouse after months at low temp.		
		1	2	3
15° to 30° C. daily alternation	1	0	36	16
	5	0	7	13
	10	0	0	0
Greenhouse	3	5	41	39
	5	2	23	17
	10	1	0	0

SUMMARY

1. Seeds of *Gentiana crinita* failed to germinate or germinated poorly
when tested on moist filter paper at a daily alternating temperature of
15° to 30° C. Poor seedling production also resulted when seeds were
planted on top of soil and placed in a greenhouse at 21° C. Pre-treatment
in a moist medium for two months at 1° or 3° C., however, was effective
in bringing about growth under the above conditions.

Further growth and development of the seedlings depended upon the
kind of soil and the temperature. The best soil used was composed of
equal parts of sand, sod soil, leaf mold, and clay with a one-inch layer of
gravel at the bottom of each pot for good drainage. The leaf mold was
found to be an essential constituent regardless of the mixture used. Pure
sand permitted no growth. For normal seedling development, a cold
period of two or three months preceding the second summer was needed.

The vitality of *Gentiana crinita* seeds was determined after storage
under various conditions. Seeds stored in open containers at room tem-
perature and 5° C. showed a complete loss of vitality after one year. Seeds
sealed in air at room temperature had also lost their vitality at this time
while those sealed in partial vacuum at room temperature and those
sealed with air and partial vacuum at 5° C. still retained their original
germination power.

2. Germination of seeds of *Gentiana andrewsii* was found to be favored

by light as well as pre-treatment at low temperatures. Treatments with 0.05 M and 0.005 M solutions of KNO_3 had no effect. Seeds of this species were not as dormant as those of fringed gentian since many seedlings appeared without low temperature pre-treatment.

3. Seeds of *Gentiana acaulis* proved entirely dependent upon low temperature pre-treatment for germination or seedling production. Two months at 1° C. or 3° C. was effective.

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CHANGE IN MINERAL COMPOSITION OF THE TOMATO PLANT IRRADIATED WITH A QUARTZ-MERCURY VAPOR LAMP AND ITS RELATION TO THE LEVEL AND RATIO OF CALCIUM AND PHOSPHORUS IN THE NUTRITIVE MEDIUM ๏

W. D. Stewart and John M. Arthur

INTRODUCTION

In a previous paper (6) it was shown that some plants when exposed to conditions of reduced light intensity and irradiated under a quartz-mercury vapor lamp responded with an increase in ash and an increase in calcium or phosphorus or both. The wave lengths in the ultra-violet active in the production of response by the plant were found to be the same as those effective in the prevention and cure of rickets, and in the activation of ergosterol—2900 to 3130 Å. Furthermore, cabbage, a plant known to be lacking in anti-rachitic properties even after prolonged irradiation, failed to show an increase in ash, calcium, or phosphorus upon irradiation. Similarity in response of the plant and animal to irradiation in the ultra-violet and lack of uniformity of this response, i.e. sometimes increase in calcium or phosphorus and other times increases in both constituents, led to an investigation of still another possibility of an analogous relation— the influence of the Ca/P ratio and the level of calcium and phosphorus of the nutritive medium upon the response of the plant to irradiation.

METHODS

Culture of plants. Tomato plants (*Lycopersicon esculentum* Mill. var. Bonny Best) were grown in soil in flats until approximately four inches in height. They were then removed, the roots well washed, and the plants transferred to paraffined corks (bored to hold two plants) placed in paraffined wooden lids over the culture vessels. The plants were held two days in tap water before use in the fractional solutions. McMurtrey's solutions (4) were used and the plants alternated at 24-hour intervals between complete minus calcium or phosphorus solutions and single salt solutions containing calcium or phosphorus. McMurtrey's "complete minus calcium" and "complete minus phosphorus" solutions were selected since with either element lacking there was no alteration in the concentration of the other essential elements, nor did precipitation occur in the preparation or use of the solutions. Since Parker (5) has shown that iron combines with the phosphate ion and renders it non-diffusible and hence non-available for the plant, iron was never added to the single salt solutions used as sources of phosphorus. Both the ratio of Ca/P and the level of calcium

and phosphorus were varied. Unless otherwise stated the ratio of calcium to phosphorus was 245:1 for the 1 p.p.m. phosphorus series, 24.5:1 for the 10 p.p.m. phosphorus, 2.4:1 for the 100 p.p.m. phosphorus, and 1:65 for the 1 p.p.m. calcium, 1:6.5 for the 10 p.p.m. calcium, and 1:0.65 for the 100 p.p.m. calcium for all of the carriers of calcium and phosphorus used except $Ca(H_2PO_4)_2$. The plants were grown on the solutions four to six weeks before harvesting.

Irradiation. Exposure of the plants took place under a Cooper Hewitt lamp 15 inches from the unshielded tube. Four irradiations of 20 or 30 seconds at 48-hour intervals were given during the last two weeks of the experiment and the plants were harvested 72 hours after the last irradiation. The characteristics of the lamp have been described by Arthur (1). At least two of the irradiations were given during periods of low light intensity.

Analyses. All plants were sampled in the afternoon, air-dried, and weighed. The tissues were ground in a "Nixtamal" mill, mixed, and any particles of metal removed with a small magnet. The material was placed in small weighing bottles and dried overnight at 100° C. About 0.5 gram of material was then weighed into tared ashing capsules (combustion capsules) and ashed in a combustion tube furnace with oxygen at 450° C. for a period of 16 hours (7). Calcium and phosphorus were determined in the same manner as described in a previous paper (6).

RESULTS

IRRADIATION OF PLANTS GROWN ON SOLUTIONS HAVING A LOW RATE OF RENEWAL

These experiments were performed in the greenhouse. Porcelain pans $12'' \times 7'' \times 2''$ were used as culture vessels and the quantity of solution limited to one liter per ten plants. A low rate of renewal of nutrient solution was used, that is, solutions were renewed at the rate of only 500 cc. per day per ten plants. The plants were alternated at 24-hour intervals between McMurtrey's complete minus phosphorus or calcium solutions and single salt solutions of different concentrations of the Ca^{++} or PO_4^{---} ions. All irradiations were given the last two weeks before sampling.

For the first test $Ca(H_2PO_4)_2$ was used as the source of calcium and phosphorus and was supplied at concentrations of 1 and 100 p.p.m. of phosphorus or calcium. Forty plants were grown five weeks on each of the cultures. One-half of the plants received four exposures of 20 seconds under the open arc. The analyses of the plants (stems and leaves) are shown in Table I. The analyses of the plants showed no response with irradiation when $Ca(H_2PO_4)_2$ was used as a source of calcium—no increase in ash, calcium, or phosphorus. Increasing the amount of calcium supplied from 1 to 100 parts per million increased the percentage ash, calcium, and

phosphorus content of the plants. When $Ca(H_2PO_4)_2$ was used as a source of phosphorus, only at a concentration of 100 p.p.m. of phosphorus was there an increase in ash upon irradiation and also an increase in phosphorus content of the plants. Increasing the amount of phosphorus supplied from 1 to 100 parts per million decreased the ash and calcium percentages but increased the percentage of phosphorus. High calcium content of plants appeared to be correlated with high calcium to phosphorus ratio of solution at this low renewal rate. Plants on solutions low in calcium, normal in phosphorus, were low in both ash and calcium, and increasing the supply of calcium increased the percentage ash, calcium, and phosphorus. Plants on solutions deficient in phosphorus, normal in calcium, had a high ash content and increasing the phosphorus in the solution decreased the ash and calcium, but increased the phosphorus content of the plants.

TABLE I

IRRADIATION OF TOMATO PLANTS; $Ca(H_2PO_4)_2$ USED AS THE SOURCE OF CALCIUM AND PHOSPHORUS. RATE OF RENEWAL OF SOLUTION ONE-HALF LITER PER DAY
PERCENTAGES ON DRY WEIGHT BASIS.* SAMPLED MARCH 22, 1934

Culture solution	% Ash		% Ca		% P$_2$O$_5$	
	Check	Irrad.	Check	Irrad.	Check	Irrad.
Complete minus Ca	15.82	15.66	0.72	0.77	3.06	2.87
1 p.p.m. Ca	10.76	10.61	0.99	1.07	2.06	1.99
100 p.p.m. Ca	13.26	12.12	1.88	1.79	3.99	3.81
1 p.p.m. P	18.25	18.41	5.51	5.23	0.44	0.51
100 p.p.m. P	15.53	17.29	3.96	3.89	2.86	3.29

* As an aid in grasping the data the more significant increases are italicized in all tables.

An experiment was made to determine the effect of the salt or compound used as a carrier of phosphorus or calcium in low concentrations. H_3PO_4, PCl_5 and $Ca(H_2PO_4)_2$ were used as the source of phosphorus and CaO, $CaCl_2$, and $Ca(H_2PO_4)_2$ for calcium. The concentrations were 1 and 10 p.p.m. of calcium or phosphorus. The plants were grown four weeks on the solutions, receiving four exposures of one minute, 36 inches from the open arc, at 48-hour intervals. Twenty plants were irradiated and 20 served as controls for each test. These plants were also grown in the greenhouse and sampled for analysis on April 26. In the low phosphorus solutions an increase in percentage ash upon irradiation was found only in case of H_3PO_4 and PCl_5 at concentrations of 10 p.p.m. accompanied by an increase in percentage calcium. Plants grown in the low calcium solutions showed a slight increase in percentage ash upon irradiation in only one salt solution —that of $Ca(H_2PO_4)_2$ at a concentration of 10 p.p.m. This was accompanied by an increase in phosphorus content. In the low phosphorus solution series increasing the amount of this element from 1 to 10 p.p.m. decreased the ash and calcium but increased the phosphorus content of the

plants. In the low calcium solution series increasing the amount of calcium from 1 to 10 p.p.m. increased the ash and calcium and, in case of $Ca(H_2PO_4)_2$ solution, the percentage phosphorus of plants. The dry weights of plant tissue produced indicate that H_3PO_4 was as good a source of phosphorus as $Ca(H_2PO_4)_2$; some injury to roots was noticed at the higher concentration but this was not reflected by any decrease in top growth. At the lower concentration PCl_5 was found to be a good source of phosphorus but marked injury to roots resulted at the higher concentration.

IRRADIATION OF TOMATO PLANTS ON SOLUTIONS HAVING A HIGH RATE OF RENEWAL

Plants grown in light cages. Four different intensities were secured by the use of cloth cages varying as to mesh; total light energy outside was considered as 100 per cent and measurements with a pyrheliometer yielded values of 78 per cent for C I, 58 per cent for C II, and 35 per cent for C III of the total energy transmitted. Each cage was approximately 7 feet high, 12 feet wide, and 60 feet long.

The containers were 8-liter glazed earthenware pots. The plants were alternated at 24-hour intervals between McMurtrey's complete minus phosphorus solutions and solutions of KH_2PO_4 containing 1, 10, and 100 p.p.m. of phosphorus. Forty plants were used for each concentration and one-half of these irradiated under the open arc four times for 30 seconds at 48-hour intervals the last two weeks before sampling. Solutions were changed as frequently as required to give a renewal rate of five liters per day per 10 plants, and the plants grown for six weeks. Pots outside were placed in a pit, the sides of which were covered with burlap to prevent the rain from spattering plants with soil. The soil in the cages was covered with straw for the same purpose. Despite attempts at insulation of the pots outside (wooden lids and straw around the pots) the temperature of the solutions on a clear day was sometimes as much as five degrees higher than in the cages, the solutions in the cages varying no more than one to two degrees Centigrade.

The dry weight data, Table II A, is of special interest as it shows that the check plants when given the maximum amount of phosphorus produced more dry weight when shaded in C I and II than those in direct sunlight. When shaded still further in C III less dry weight was produced. Although the plants received three irradiations during periods of cloudy weather no apparent injury resulted from the heavy dosage to the plants grown outside; in fact there is some evidence of increased dry weight production. In C II and C III, however, the plants were definitely injured by irradiation, which was evinced by "shine" and curling of the leaves as well as by decrease in dry weight. This may be seen in Figure 1 A, B, C, and

D. The effect of decreased light intensity in greatly increasing the injury of plants to ultra-violet irradiation has been discussed in a previous publication (6). Increasing the phosphorus concentration greatly increased the dry weight produced.

TABLE II

CHECK AND IRRADIATED TOMATO PLANTS GROWN IN CLOTH CAGES. INCREASING AMOUNTS OF PHOSPHORUS SUPPLIED AS KH₂PO₄. RATE OF RENEWAL OF SOLUTION FIVE LITERS PER DAY. SAMPLED AUGUST 7, 1934

A. Dry weights of whole plants in grams (to nearest whole numbers)

	1 p.p.m. of P		10 p.p.m. of P		100 p.p.m. of P	
	Check	Irrad.	Check	Irrad.	Check	Irrad.
Outside	38	38	71	75	64	78
Cage I	43	43	66	67	82	76
Cage II	31	23	75	64	81	69
Cage III	39	31	54	50	62	45

B. Analyses of leaves. Dry weight basis

	% Ash		% Ca		% P₂O₅	
	Check	Irrad.	Check	Irrad.	Check	Irrad.
Outside —1 p.p.m. P	14.11	14.12	2.06	2.03	0.68	0.67
Cage I —1 p.p.m. P	13.60	14.09	1.80	1.96	0.53	0.55
Cage II —1 p.p.m. P	13.05	12.25	2.03	2.04	0.51	0.53
Cage III —1 p.p.m. P	14.05	14.38	1.81	2.08	0.52	0.59
Outside —10 p.p.m. P	12.86	13.34	2.23	2.42	1.13	1.19
Cage I —10 p.p.m. P	10.76	13.16	1.77	2.28	1.03	1.27
Cage II —10 p.p.m. P	12.25	13.90	1.97	2.11	1.14	1.26
Cage III —10 p.p.m. P	12.70	14.56	2.19	2.30	1.26	1.37
Outside —100 p.p.m. P	12.49	13.99	2.11	2.45	1.35	1.62
Cage I —100 p.p.m. P	12.41	14.40	2.02	2.45	1.45	1.66
Cage II —100 p.p.m. P	12.88	13.75	2.03	2.13	1.52	1.68
Cage III —100 p.p.m. P	13.83	14.47	1.99	2.21	1.77	1.88

C. Analyses of stems. Dry weight basis

	% Ash		% Ca		% P₂O₅	
	Check	Irrad.	Check	Irrad.	Check.	Irrad.
Outside —1 p.p.m. P	14.20	15.92	1.99	2.09	0.41	0.39
Cage I —1 p.p.m. P	15.07	15.30	1.73	1.87	0.42	0.40
Cage II —1 p.p.m. P	14.32	13.56	1.69	2.00	0.42	0.39
Cage III —1 p.p.m. P	16.51	16.44	1.65	1.74	0.40	0.44
Outside —10 p.p.m. P	13.56	14.08	1.56	1.63	1.08	1.10
Cage I —10 p.p.m. P	13.73	15.70	1.12	1.49	1.04	1.09
Cage II —10 p.p.m. P	17.34	18.28	1.34	1.36	1.01	1.03
Cage III —10 p.p.m. P	18.49	20.11	1.46	1.51	1.04	1.30
Outside —100 p.p.m. P	18.05	18.25	1.49	1.51	1.45	1.69
Cage I —100 p.p.m. P	16.48	19.11	1.39	1.53	1.51	1.73
Cage II —100 p.p.m. P	18.73	20.84	1.30	1.40	1.53	1.73
Cage III —100 p.p.m. P	19.08	20.44	1.39	1.42	1.72	1.69
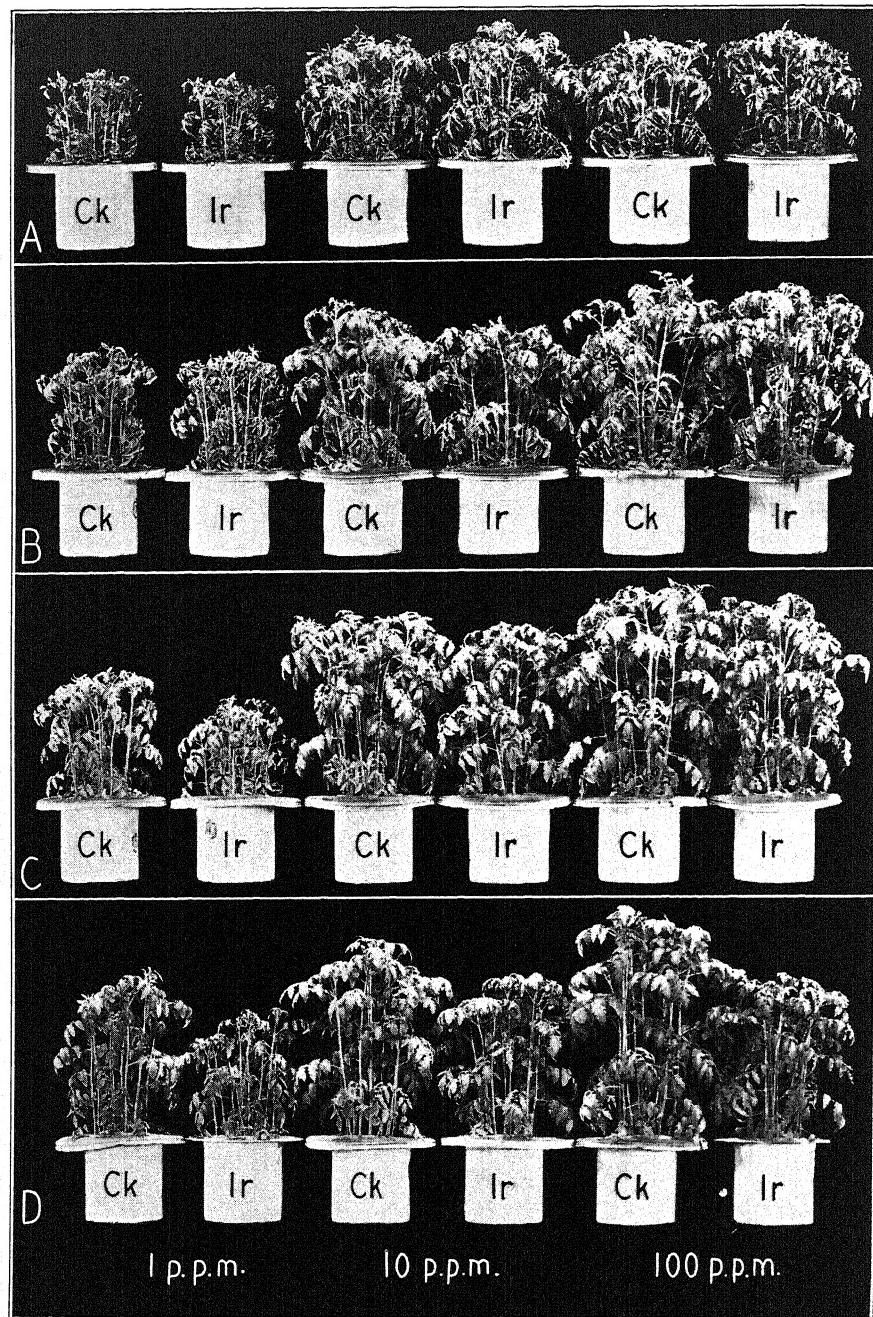
FIGURE 1. Effect of irradiating tomato plants in solutions of KH_2PO_4 containing 1, 10, and 100 p.p.m. phosphorus, at different light intensities. (A) Grown outside, (B) grown in Cage I, (C) in Cage II, and (D) in Cage III. Note the marked injury of irradiation at the lower light intensities and lower phosphorus concentrations.

Analyses of the leaves for ash, calcium, and phosphorus yielded the data in Table II B. Even with a high rate of renewal of solution only two groups of plants on the 1 p.p.m. concentrations of solution showed a slight increase in ash with irradiation and this was accompanied by an increase in calcium. Leaves of plants in all of the cages on the solutions containing 10 p.p.m. phosphorus responded to irradiation with increases of ash, calcium, and phosphorus; the same type of response was had also with leaves from all the cages on the solutions of 100 p.p.m. concentration of phosphorus. Comparison of the phosphorus contents of the leaves of the plants shows increase in phosphorus as the concentration of KH_2PO_4 increases. The calcium content of the whole plant was not altered by the increase in phosphorus when the phosphorus was supplied as KH_2PO_4 but this element increased slightly with phosphorus concentration in the leaves and decreased in the stems. For plants grown on the solutions of 100 p.p.m. phosphorus, the phosphorus content was lowest outside and gradually increased with decrease of light intensity.

Determinations of ash, calcium, and phosphorus of the stems yielded the results given in Table II C. With the 1 p.p.m. concentration of phosphorus, stems of irradiated plants were higher in calcium than those of the non-irradiated and in two cases appeared to be higher in ash. At concentrations of 10 p.p.m. of phosphorus the response to irradiation was increase in ash and calcium for the stems of plants outside and in Cage I, increase in ash but no change in calcium or phosphorus in Cage II, and in Cage III increase in ash and phosphorus. For concentrations of 100 p.p.m. of phosphorus all stems of irradiated plants showed increases of ash and phosphorus except Cage III where there was an increase of ash but no alteration of calcium or phosphorus content. Here again increase in concentration of phosphorus in the nutrient solution was accompanied by increase in phosphorus content of the plant. In general, ash and phosphorus percentages increased and calcium percentages decreased with increasing concentrations of phosphorus. There was a definite increase in ash with decreasing light intensity. This is apparent if the ash figures for each light condition be averaged. The values for ash so calculated are: Outside, 11.45; Cage I, 11.32; Cage II, 12.59; Cage III, 13.52 for the check plants and 12.06, 12.50, 13.16, and 14.24 respectively for the irradiated plants. Similarly it may be calculated that there is some indication of a slight increase in phosphorus and a decrease in calcium with decreasing light intensity. A summary of correlations of constituents with various factors calculated in this way follows:

With:	Dry Weight	Ash	Ca	P_2O_5
Decreasing light intensity	Increase to C II then decrease	Increase	Decrease	Increase
Increasing phosphorus concentration of nutrient solution	Increase	{Leaves ? {Stems, increase	{Leaves, increase {Stems, decrease	Increase
Ultra-violet irradiation	Decrease	Increase	Increase	Increase

The response of the roots to irradiation was very similar to that of the stems in that percentage ash was increased. There were no increases, however, of either calcium or phosphorus upon irradiation. The percentage phosphorus increased as the concentration of the element was increased in the medium but without alteration of the calcium content. Light intensity did not seem to affect either calcium, phosphorus, or ash content of the roots.

In Table III are comparisons of different methods of securing values for the estimation of mineral content. Samples were ashed in a muffle at 650° C. for 16 hours, others were ashed in the tube furnace at 450° C. for

TABLE III

IRRADIATION OF TOMATO PLANTS GROWN IN CLOTH CAGES. PHOSPHORUS AS KH_2PO_4. SAMPLED AUGUST 7, 1934. COMPARISON OF METHODS FOR EVALUATING MINERAL CONTENT

		Leaves % ash			Stems % ash			Roots % ash		
		Muffle	Tube	H₂SO₄	Muffle	Tube	H₂SO₄	Muffle	Tube	H₂SO₄
Outside— 1 p.p.m. P	Ck.	10.92	14.11	16.94	10.35	14.20	17.95	8.99	12.48	14.92
	Irr.	10.94	14.12	17.28	11.78	15.92	19.51	9.60	14.10	15.37
Cage I— 1 p.p.m. P	Ck.	10.51	13.60	16.53	11.39	15.07	18.29	8.89	11.47	13.69
	Irr.	11.46	14.09	17.45	11.50	15.30	18.89	9.60	11.42	14.74
Cage II— 1 p.p.m. P	Ck.	9.98	13.05	16.39	10.91	14.32	17.01	8.48	9.97	12.67
	Irr.	9.83	12.25	16.11	10.03	13.56	16.86	7.85	8.96	12.44
Cage III— 1 p.p.m. P	Ck.	11.39	14.05	16.73	13.25	16.51	19.75	8.92	10.96	13.84
	Irr.	12.34	14.38	18.64	13.40	16.44	20.64	9.62	11.45	15.08
Outside— 10 p.p.m. P	Ck.	10.97	12.86	16.21	11.24	13.56	16.39	10.52	12.79	15.67
	Irr.	11.30	13.34	17.27	11.73	14.08	18.93	11.70	14.02	17.27
Cage I— 10 p.p.m. P	Ck.	8.82	10.76	13.87	11.00	13.73	15.57	7.86	10.24	11.72
	Irr.	10.78	13.16	16.85	12.62	15.70	18.57	9.47	12.12	14.08
Cage II— 10 p.p.m. P	Ck.	10.14	12.25	15.32	13.58	17.34	18.91	9.49	10.66	13.18
	Irr.	11.50	13.90	17.78	14.70	18.28	19.74	10.67	12.25	14.92
Cage III— 10 p.p.m. P	Ck.	11.37	12.70	15.61	14.15	18.49	20.49	9.55	11.40	13.89
	Irr.	12.87	14.56	18.02	15.42	20.11	21.04	10.68	13.14	15.36
Outside— 100 p.p.m. P	Ck.	10.81	12.49	16.00	11.66	18.05	17.13	13.44	15.42	17.62
	Irr.	11.80	13.99	18.41	14.23	18.25	20.33	14.10	15.81	17.67
Cage I— 100 p.p.m. P	Ck.	11.05	12.41	16.20	12.98	16.48	18.09	12.98	14.17	16.39
	Irr.	12.97	14.40	18.63	15.36	19.11	22.10	13.14	14.20	16.80
Cage II— 100 p.p.m. P	Ck.	11.65	12.88	17.09	14.65	18.73	20.50	12.20	13.49	16.05
	Irr.	12.25	13.75	18.60	16.29	20.84	21.96	13.58	15.14	17.31
Cage III— 100 p.p.m. P	Ck.	13.18	13.83	17.65	16.46	19.08	21.70	11.82	14.04	16.70
	Irr.	13.67	14.47	19.82	17.18	20.44	23.55	13.45	14.89	17.56
Relative average values with tube values equal to 100		84.9	100	127.3	78.1	100	115.0	84.3	100	119.9

16 hours in the presence of oxygen, and still others were "wet" with H_2SO_4 and ashed at 650° C. for 16 hours in the muffle. In a former publication (7) the relative merits of the three methods were discussed. As already reported the values for the muffle determination of ash at the high temperature are lower due to loss of volatile inorganic constituents. The higher values obtained by ashing with sulphuric acid are due to the partial conversion of basic constituents to sulphates with a resulting increase in molecular weight. The ash percentages obtained by the use of the tube are the most reliable since comparable values may be had from combustions carried on at widely separated times and ashing by this method produces the least change in ash constituents. The differences in percentage ash between irradiated and non-irradiated or check plants are at once apparent whether the muffle, tube, or sulphuric acid methods of ashing are followed.

Potassium was estimated by the cobaltinitrite method (3) upon ashing the material with sulphuric acid and ferric sulphate (2). The tabulated values of this element obtained for tissues of irradiated and non-irradiated plants are omitted. Much experience with this method and a more thorough acquaintance with its faults have led to the belief that the method is unreliable and should not be used. The results indicate strongly the probability of increase in potassium content with irradiation and with decreasing light intensity when phosphorus is supplied as KH_2PO_4 in concentrations of 10 and 100 p.p.m.

First series of plants grown in greenhouse. An experiment was planned to test the influence of the ion carrying phosphorus upon response to irradiation when supplied at the higher rate of renewal for concentrations of 1, 10, and 100 p.p.m. of phosphorus. H_3PO_4, $Ca(H_3PO_4)_2$, KH_2PO_4, NaH_2PO_4, and $MgHPO_4$ were the salts used as sources of phosphorus. As usual the plants were alternated at 24-hour intervals between McMurtrey's complete minus phosphorus solutions and the single salt solutions containing phosphorus. The plants grew five weeks on the solutions, receiving four irradiations of 30 seconds the last two weeks before sampling. Forty plants were used for each concentration; 20 were irradiated and 20 served as controls.

The dry weights of leaves and stems are shown in Table IV A and the analyses of leaves and stems for ash, calcium, and P_2O_5 in Table IV B and C. At this level of supply the growth of the plants as shown by the dry weight produced was good even at concentrations of 1 p.p.m. of phosphorus, and the growth was slightly better on H_3PO_4 solution than on $Ca(H_3PO_4)_2$ at lowest concentration, but with concentrations of 10 p.p.m. of P as H_3PO_4 the roots of the plants were badly injured and top growth very poor; this is in contrast with the excellent growth at the same concentration with the low rate of renewal described above. Growth of the

plants on solutions of 10 p.p.m. phosphorus was very similar to that on concentrations of 100 p.p.m. of phosphorus for all of the other salts used as sources of phosphorus.

In Table IV B are data from the analyses of the leaves. Response to

TABLE IV

CHECK AND IRRADIATED TOMATO PLANTS GROWN IN GREENHOUSE IN SOLUTIONS WITH DIFFERENT CARRIERS OF PHOSPHORUS. SAMPLED DECEMBER 8, 1934

A. Dry weights of leaves and stems in grams (to nearest whole numbers)

Carrier	1 p.p.m.		10 p.p.m.		100 p.p.m.	
	Check	Irrad.	Check	Irrad.	Check	Irrad.
Phosphorus as H_3PO_4	27	25	2	2	—	—
Phosphorus as CaH_2PO_4	25	21	35	36	38	34
Phosphorus as KH_2PO_4	32	26	42	42	41	39
Phosphorus as NaH_2PO_4	29	25	43	39	38	35
Phosphorus as $MgHPO_4$	—	—	38	34	—	—

B. Analyses of leaves. Dry weight basis

	% Ash		% Ca		% P_2O_5	
	Check	Irrad.	Check	Irrad.	Check	Irrad.
1 p.p.m. P as NaH_2PO_4	20.15	22.19	1.07	1.19	0.84	1.01
1 p.p.m. P as H_3PO_4	16.97	21.79	0.96	1.12	0.83	1.04
1 p.p.m. P as KH_2PO_4	20.18	19.80	1.03	1.14	0.80	0.93
1 p.p.m. P as $Ca(H_2PO_4)_2$	19.50	21.05	1.02	1.08	0.67	0.77
10 p.p.m. P as NaH_2PO_4	15.98	17.01	2.22	2.30	2.02	2.13
10 p.p.m. P as $MgHPO_4$	15.69	16.72	2.79	2.90	2.00	2.10
10 p.p.m. P as KH_2PO_4	14.50	17.59	2.15	2.44	1.77	2.05
10 p.p.m. P as $Ca(H_2PO_4)_2$	16.31	15.67	2.28	2.41	1.75	1.97
100 p.p.m. P as NaH_2PO_4	14.43	16.37	2.29	2.40	2.47	2.72
100 p.p.m. P as KH_2PO_4	14.32	16.76	2.35	2.36	2.43	2.70
100 p.p.m. P as $Ca(H_2PO_4)_2$	16.70	15.88	2.42	2.74	2.79	2.68
Entire plant 10 p.p.m. P. as H_3PO_4	15.91	16.31	4.33	4.16	1.51	1.56

C. Analyses of stems. Dry weight basis

	% Ash		% Ca		% P_2O_5	
	Check	Irrad.	Check	Irrad.	Check	Irrad.
1 p.p.m. P as NaH_2PO_4	23.00	23.80	2.22	2.17	0.373	0.397
1 p.p.m. P as KH_2PO_4	23.52	24.45	2.40	2.38	0.403	0.419
1 p.p.m. P as $Ca(H_2PO_4)_2$	24.74	25.10	2.23	2.15	0.337	0.348
1 p.p.m. P as H_3PO_4	24.18	25.18	2.22	2.53	0.419	0.413
10 p.p.m. P as NaH_2PO_4	25.39	26.75	2.25	2.07	1.54	1.65
10 p.p.m. P as KH_2PO_4	24.96	26.32	2.01	2.19	1.59	1.52
10 p.p.m. P as $Ca(H_2PO_4)_2$	24.92	26.30	2.47	2.35	1.37	1.58
10 p.p.m. P as $MgHPO_4$	24.29	24.56	2.16	2.25	1.50	1.55
100 p.p.m. P as NaH_2PO_4	25.07	26.10	2.07	2.15	2.24	2.27
100 p.p.m. P as KH_2PO_4	25.50	26.28	1.82	1.86	2.14	2.22
100 p.p.m. P as $Ca(H_2PO_4)_2$	26.37	26.66	2.38	2.54	2.11	2.19

irradiation occurred even at concentrations of 1 p.p.m. of phosphorus at this level of supply, and the response was increase in ash (KH_2PO_4 excepted) and increase in calcium and phosphorus except for plants on $Ca(H_2PO_4)_2$ where the increase in ash, as usual for low concentrations of phosphorus as $Ca(H_2PO_4)_2$, was accompanied by an increase in phosphorus.

At concentrations of 10 p.p.m. of phosphorus, irradiation resulted in increase in ash, except for $Ca(H_2PO_4)_2$, and this was associated with increase in calcium and phosphorus. At concentrations of 100 p.p.m. of phosphorus there was an increase in ash with irradiation for plants on NaH_2PO_4 and KH_2PO_4 but none for those on the $Ca(H_2PO_4)_2$, with the customary tendency toward greater increase of phosphorus than of calcium; and with the $Ca(H_2PO_4)_2$ the shift to increase in calcium with no change in phosphorus. Also to be noted is the decrease in ash and increase in phosphorus and calcium that occurred as the concentration of phosphorus was raised from 1 to 10 p.p.m.

The results from the analyses of the stems in Table IV C did not yield evidence of any effect of irradiation on composition of this tissue other than a slight increase in ash of the irradiated plants. Phosphorus content increased with increase in concentration of this element; calcium content was not affected by the increase in phosphorus content for any of the carriers of phosphorus except KH_2PO_4. Increase in phosphorus was associated here with a slight decrease in calcium content. Ash content of the stems increased as the concentration of phosphorus was raised from 1 to 10 p.p.m., but further increase in concentration to 100 p.p.m. did not raise the level of ash content except for the $Ca(H_2PO_4)_2$.

Second series of plants grown in greenhouse. In the next series of tests to be described McMurtrey's complete minus phosphorus solutions were used diluted to one-half the usual concentration. Such dilutions contained 122 p.p.m. of calcium. The single salt solutions used as sources of phosphorus were NaH_2PO_4, KH_2PO_4, and $Ca(H_2PO_4)_2$ at concentrations of 1.22, 12.2, and 122 p.p.m. of phosphorus. Hence the ratios of calcium to phosphorus were 100:1 for the 1.22 p.p.m., 10:1 for the 12.2 p.p.m., and 1:1 for the 122 p.p.m. of phosphorus. As with the previous tests, the plants were alternated at 24-hour intervals between the complete minus phosphorus solutions and the single salt solutions serving as the source of phosphorus. Forty plants were grown for each condition; 20 received four irradiations of 30 seconds 15 inches from the open arc given at 48-hour intervals the last two weeks before sampling, and 20 remained untreated. The plants were grown four weeks before sampling.

Values for the dry weights of leaves and stems per 20 plants are given in Table V A. Plants receiving NaH_2PO_4 attained maximum dry weight production on the concentration of 122 p.p.m. of phosphorus but those on the solutions of KH_2PO_4 and $Ca(H_2PO_4)_2$ reached their maxima at the

12.2 p.p.m. level, although the differences in dry weights at concentrations of 12.2 and 122 p.p.m. were slight in all three cases. The minimum dry weight was obtained at the concentrations of 1.22 p.p.m. for all three salts

TABLE V

Check and Irradiated Tomato Plants Grown in Greenhouse. Second Test. Sampled February 18, 1935

A. Dry weights of leaves and stems in grams (to nearest whole numbers)

Carrier	1.22 p.p.m.		12.2 p.p.m.		122.0 p.p.m.	
	Check	Irrad.	Check	Irrad.	Check	Irrad.
Phosphorus as NaH_2PO_4	23	23	30	31	40	33
Phosphorus as KH_2PO_4	30	21	41	40	40	34
Phosphorus as $Ca(H_2PO_4)_2$	29	26	56	53	47	46

B. Analyses of leaves. Dry weight basis

	% Ash		% Ca		% P_2O_5	
	Check	Irrad.	Check	Irrad.	Check	Irrad.
1.22 p.p.m. P as NaH_2PO_4	10.47	14.83	1.84	2.32	0.41	0.58
1.22 p.p.m. P as KH_2PO_4	11.58	13.16	1.91	2.57	0.45	0.63
1.22 p.p.m. P as $Ca(H_2PO_4)_2$	12.51	14.89	2.05	2.48	0.46	0.54
12.2 p.p.m. P as NaH_2PO_4	12.11	13.90	2.16	2.55	1.34	1.56
12.2 p.p.m. P as KH_2PO_4	13.21	14.59	2.18	2.54	1.52	1.65
12.2 p.p.m. P as $Ca(H_2PO_4)_2$	14.78	14.25	2.54	2.50	1.68	1.74
122.0 p.p.m. P as NaH_2PO_4	12.10	13.27	2.11	2.32	1.91	2.04
122.0 p.p.m. P as KH_2PO_4	12.73	14.31	2.25	2.58	1.95	2.06
122.0 p.p.m. P as $Ca(H_2PO_4)_2$	13.14	14.63	2.61	2.85	1.90	2.22

C. Analyses of stems. Dry weight basis

	% Ash		% Ca		% P_2O_5	
	Check	Irrad.	Check	Irrad.	Check	Irrad.
1.22 p.p.m. P as NaH_2PO_4	15.37	17.61	2.03	2.13	0.39	0.42
1.22 p.p.m. P as KH_2PO_4	15.95	17.35	1.86	2.20	0.38	0.46
1.22 p.p.m. P as $Ca(H_2PO_4)_2$	17.32	17.71	2.07	2.18	0.36	0.37
12.2 p.p.m. P as NaH_2PO_4	17.18	18.26	1.90	2.04	1.16	1.18
12.2 p.p.m. P as KH_2PO_4	19.27	19.10	1.95	1.92	1.36	1.32
12.2 p.p.m. P as $Ca(H_2PO_4)_2$	19.68	19.27	2.14	2.14	1.46	1.52
122.0 p.p.m. P as NaH_2PO_4	16.34	16.05	1.71	1.67	1.75	1.79
122.0 p.p.m. P as KH_2PO_4	18.64	20.13	1.61	1.80	1.73	1.75
122.0 p.p.m. P as $Ca(H_2PO_4)_2$	16.47	19.71	2.12	2.48	1.76	2.06

of phosphorus. The poorest source of phosphorus as indicated by dry weight production was NaH_2PO_4, and the best was $Ca(H_2PO_4)_2$.

In Table V B the analyses of the leaves are given. Increase in ash occurred at all concentrations of phosphorus for plants on the solutions of NaH_2PO_4 and KH_2PO_4 with irradiation, and this increase was accompanied by increase of calcium and phosphorus. Irradiation of the plants on

$Ca(H_2PO_4)_2$ resulted in increase in ash, calcium, and phosphorus at concentrations of 1.22 and 122 p.p.m. of P, but irradiation of the plants on the solutions containing 12.2 p.p.m. of phosphorus did not alter the ash, calcium, or phosphorus content of the leaves. Phosphorus content of leaves increased as the concentration of phosphorus was raised, and content of calcium increased slightly. Upon increasing the concentration of phosphorus from 1.22 to 12.2 p.p.m. the ash content of control plants increased, but further increase in concentration of phosphorus gave a decrease of ash or else no change. This trend was not apparent in the irradiated plants. Ash content was low at all concentrations of phosphorus as NaH_2PO_4, slightly higher for KH_2PO_4, and highest for the $Ca(H_2PO_4)_2$.

Table V C shows the results from the analyses of the stems. Irradiated stems were higher in ash and calcium than the untreated at 1.22 p.p.m. of phosphorus for all three of the salts used. At 12.2 p.p.m. of phosphorus only the NaH_2PO_4-plants showed an increase in ash and calcium. Ash, calcium, and phosphorus content of stems of plants on the KH_2PO_4 and $Ca(H_2PO_4)_2$ salt solutions remained unaltered by irradiation at this concentration. At concentrations of 122 p.p.m. of phosphorus the NaH_2PO_4-plants failed to respond to irradiation but the plants on KH_2PO_4 showed increase in ash and calcium and those on the $Ca(H_2PO_4)_2$ increase in ash, calcium, and phosphorus. Here again raising the concentration of phosphorus from 1.22 to 12.2 p.p.m. resulted in an increase in ash content of control plants, and further increase to 122 p.p.m. was associated with a decrease. Phosphorus content of the stems increased as the concentration of the carrier was increased, and the calcium content was unchanged except for the NaH_2PO_4 group, the calcium content of which appeared to decrease as the phosphorus increased.

In order to conserve space, tabular data from analyses of the roots are omitted. The content of ash, calcium, and phosphorus was not altered by irradiation at concentrations of 1.22 and 12.2 p.p.m. of phosphorus. At the 122 p.p.m. level a decrease in ash was noticed for the NaH_2PO_4 and the KH_2PO_4 and also a decrease in phosphorus. Phosphorus content of the roots increased as the concentration was raised in the solutions but the calcium contents were not changed.

DISCUSSION OF RESULTS

It has already been shown that not all plants respond to irradiation, and that several factors condition the presence or absence of response (6). Level of light intensity at which plants were grown preceding irradiation was the most important of these. Unless the plants were grown under conditions of reduced light intensity or exposed for a period of 24 to 48 hours at low light intensity preceding irradiation no increase in ash, calcium, or phosphorus of the plant resulted from such irradiation under the

quartz-mercury vapor lamp. Results from some of the previous tests indicated that nutritional factors affected the response. The rôle of calcium and phosphorus as related to the response of the tomato plant to irradiation is the object of the experiments described in this paper.

The nutritional factors governing the response of the plant to irradiation are difficult to separate because of the reciprocal relationships existing between certain of the ions. McMurtrey's "complete minus calcium" and "complete minus phosphorus" solutions were found to be exceptionally well adapted for fractional solution studies and hence excellent for studying the rôle of calcium and phosphorus in the response of the tomato plant to irradiation. The advantage of these solutions is that either calcium or phosphorus may be omitted without changing either the ratio or the concentration of the other essential elements.

The level of supply of calcium or phosphorus in the nutritive media of the plant, as with the animal, is more important than the ratio of calcium to phosphorus in determining response to irradiation. If either calcium or phosphorus is omitted from the nutrient solution, or if the level of supply is low, irradiation of the plant fails to produce increase in ash, calcium, or phosphorus contents of such plants. However, on raising the level of supply of either calcium or phosphorus (higher rate of solution renewal), with the same ratio of calcium to phosphorus, irradiation of the plant results in an increase of ash and calcium, or phosphorus, or both. With a high ratio of calcium to phosphorus and a low level of supply of phosphorus the response to irradiation is increase in ash and calcium instead of increase in ash and phosphorus; with a reverse in ratio and with calcium deficient the response is increase in ash and phosphorus. As the ratio of calcium to phosphorus decreases, with high rate of renewal, increase in phosphorus becomes more evident on irradiation and increase in calcium less frequent. Interpretation is difficult, however, since in these cases reciprocal relations between calcium and the carriers of phosphorus (Na, K, Mg) may be responsible for depression of calcium as these ions tend to repress absorption of calcium. This does not preclude the alternative explanation that increase in phosphorus supply lowers calcium absorption (Table I). With a low level of supply of phosphorus and a high ratio of calcium to phosphorus in the nutrient solution the content of calcium of the plant is high and that of phosphorus low; increase in concentration of phosphorus raises the content of phosphorus in the plant and lowers the content of calcium. If calcium is the deficient element the phosphorus content is high and increase in calcium lowers the phosphorus content of the plant and raises the calcium. But this reciprocal relationship is not so apparent where the level of supply is high.

With a low level of supply of phosphorus (Table I) tomato plants grown on phosphorus-deficient solutions were higher in ash, and raising the con-

centration of phosphorus increased the content of phosphorus of the plant but lowered the ash content. Phosphorus content of tomato plants increased as the concentration of the element was raised in the solution. Parker (5) has already demonstrated this for corn and soybean. The calcium content of the tomato plant also increases with increase of the element in the nutrient solution.

An average of all of the values given in Table II for each constituent shows that dry weight increases with shading down to a certain value, increases also with increasing phosphorus concentration of solution, but decreases with ultra-violet irradiation when plants are shaded. Ash increases with shading in the whole plant and with phosphorus concentration (only in stems) and also increases with irradiation. Calcium in plants decreases with shading, increases in leaves with increasing phosphorus concentration of solution, but decreases in stems. Calcium increases with irradiation. Phosphorus in plants increases with shading, with phosphorus concentration of solution, and with irradiation. These plants were grown during the summer so that light intensity was much higher outside than in the greenhouse where those plants represented in Table I were grown and sampled during March. Probably only in Cage III, Table II, were the light intensities at all comparable with the level of intensity available during the tests in March.

At a concentration of 1 p.p.m., phosphoric acid was as good a source of phosphorus as the salts of phosphoric acid. The tomato plant was not injured by this concentration of the hydrogen ion but injury was observed at concentrations of 10 p.p.m. at the higher level of supply. With the low level of supply only slight injury to the roots occurred. Apparently the hydrogen or hydroxyl ions are satisfactory carriers of nutritive ions for this plant.

SUMMARY

Tomato plants were grown outside, in the greenhouse, and under shading cloth on fractional solution cultures. The plants were alternated at 24-hour intervals between McMurtrey's "complete minus phosphorus or calcium" solutions and single salt solutions containing calcium or phosphorus or both. Rate of supply of calcium and phosphorus and ratio of calcium to phosphorus were varied. The plants were grown four to six weeks on these solutions and their response to irradiation under a quartz-mercury vapor lamp as reflected by change in ash, phosphorus, and calcium content observed. Results from the data are summarized.

1. Plants grown on solutions lacking calcium or phosphorus showed no increase in ash, calcium, or phosphorus on irradiation.

2. Level of supply of calcium or phosphorus and not ratio of calcium to phosphorus, determines presence or absence of response to irradiation.

Without altering the ratio of calcium to phosphorus (high ratios) presence or absence of response was secured by controlling the rate of solution renewal.

3. Plants grown on solutions deficient in phosphorus (low renewal rate) were high in ash. Increase in the concentration of phosphorus of the solution lowered the ash content.

4. Decreasing light intensity during the summer increased dry weight, ash, and phosphorus content but decreased calcium.

5. On solutions deficient in phosphorus (high ratio of calcium to phosphorus) the response to irradiation was increase in ash and calcium whereas on solutions deficient in calcium (high phosphorus-low calcium) the response was increase in ash and phosphorus. With intermediate values for ratios of calcium to phosphorus the response was increase in ash, calcium, and phosphorus.

6. A reciprocal relationship between calcium and phosphorus was observed.

7. Phosphoric acid at concentrations of 1 p.p.m. was an excellent source of phosphorus for the tomato plant.

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BIONOMICS OF THE PLUM AND PEACH LEAFHOPPER, MACROPSIS TRIMACULATA

ALBERT HARTZELL

The plum and peach leafhopper, *Macropsis trimaculata* (Fitch), is one of the most serious insects affecting the peach (*Prunus persica* [L.] Stokes) in northeastern United States and in southern Canada. Through its feeding activities it transmits peach yellows (14) and little peach (17, 21) two very important diseases of the peach throughout the areas in which they are endemic. It also transmits yellows (20) and little peach from cultivated plums (*Prunus salicina* and *Prunus simonii*) to peach. The relation of this insect to peach yellows has been discussed by the writer in previous publications (10, 11, 12). The present paper will be restricted therefore to a description of the insect, of which the early stages are here described and illustrated for the first time, and especially to the life history and habits of this unusual species of leafhopper. In spite of the fact that this species was described nearly a century ago, very little was known of its life history when this investigation was begun. The lack of knowledge was no doubt due to the fact that the species was overlooked except by persons interested in taxonomy. This neglect was due at least in part to its peculiar habits of concealment and also to the fact that except in favored localities it is somewhat rare.

The habits of *Macropsis trimaculata* in many ways resemble those of a treehopper. The adults when disturbed are rarely seen to fly nor do the nymphs hop. In place of feeding on the leaves as in the usual habit of leafhoppers, this species feeds on twigs and is seldom observed on the leaves. Furthermore while its chief visible damage is done indirectly to peach by transmitting yellows, it occurs in greatest numbers on plum and is found only sparingly on peach. The symptoms of yellows on plum are masked and therefore until recently have been overlooked. The double host relationship has suggested to the writer that an appropriate common name for *Macropsis trimaculata* would be the plum and peach leafhopper, which name was proposed in a previous publication (10). The unusual habits of this insect as stated above and the specific conditions required for its development have made the study of this species difficult. Except for a species of spider feeding on a nymph, no natural enemies were observed.

The work[1] has extended over a number of years and was done in conjunction with the study of the transmission of peach yellows. While certain

details of the life history need further investigation, it is believed that the essential features of the bionomics of this species are as stated here.

The plum and peach leafhopper was first described in 1851 by Fitch (6), under the scientific name *Pediopsis trimaculatus*. Since then the insect has been redescribed under several scientific names by various authors, but is now designated as *Macropsis trimaculata* (Fitch). Breakey (3) has published recently a review of the genus *Macropsis*. For the taxonomic literature prior to the above named review, the reader is referred to Van Duzee's Catalogue of Hemiptera of America north of Mexico (35). As the species is variable both as to size and color markings, it is possible that future taxonomic study may change the present conception regarding its status.

DISTRIBUTION

Macropsis trimaculata is widely distributed throughout the Transitional and Upper Austral life zones in eastern North America. The species has been reported from the provinces of Quebec (28, 34, 35) and Ontario (9), Canada, and in the United States from Maine (25), New York (16, 24, 32, 33), Pennsylvania (36), Michigan (31), Iowa (23, 27), Kansas (31), and Colorado (1). The insect is known to occur also in Connecticut (4), New Jersey (14), Delaware, Maryland (20), Virginia (29), Ohio (3, 26), Indiana (15), and Illinois (20). It is difficult in reviewing the literature to determine in every instance whether a given locality record refers to this species or to a closely related species. The writer has followed the classification as given in Van Duzee's Catalogue of Hemiptera (35).

A survey made by T. F. Manns and M. M. Manns (20) beyond the climatic range of peach and Japanese plum in Minnesota and North Dakota showed no *Macropsis trimaculata* on wild plum. This led them to conclude that the species was introduced probably on Japanese or Chinese plums, and has not reached as yet the outer limit of the range of wild plum, since it has been reported from Ontario and Quebec, areas almost as severe in climate. The writer (10) has called attention to the fact that the range of this species is not coextensive with *Prunus americana* Marsh., its principal wild host. Its southern range corresponds with the southern limit of peach yellows, although *Prunus americana* extends much farther southward. So far as is known *Macropsis trimaculata* is confined to North America, although this does not exclude the possibility that a careful study of the leafhoppers of eastern Asia may show the species to be Oriental in origin.

HOST PLANTS

Extensive surveys made by the writer in Connecticut, New York, New Jersey, Pennsylvania, and Delaware show that *Macropsis trimaculata* lives principally on plum. *Prunus americana* is its chief wild host, although it has been reported from *Prunus angustifolia*, *Prunus munsoniana* (18,

19), and *Prunus pissardi* (4). The writer (10) has previously called attention to the positive correlation of *Macropsis trimaculata* to *Prunus americana* and the incidence of yellows in peach orchards adjacent to woodland. According to Manns (20) it prefers Japanese and Chinese plums (*Prunus salicina* and *P. simonii*), especially such varieties as Abundance, Red June, Chalco, Chabot, Satsuma, and Santa Rosa. It also occurs on *Prunus domestica* in considerable numbers. It was first reported on peach in 1927, from Virginia (29). Kunkel (14) found it more numerous on peach than on plum in the fore part of summer in the vicinity of Yonkers, New York. Late in the season he reports that the reverse is true. When adults, collected July 1 from *Prunus americana*, in the present investigation were given their choice of feeding on wild plum or on peach twigs, 60 per cent preferred peach twigs. On the other hand the mortality of nymphs reared on peach twigs was found to be considerably higher than on plum. There is a record from grape by LaHue (15). The insect was collected on apricot (*Prunus armeniaca* L.), July 24, 1937, by the writer at Lebanon, Pennsylvania.

DESCRIPTION OF THE INSECT

Adult. The adults[2] of *Macropsis trimaculata* are of a dull reddish-brown color (Fig. 1 A, B). The males are usually darker and somewhat smaller than the females. The markings are rather indistinct, but well marked specimens have three transparent white spots in a row on each elytron. The average length of the females is 5 mm. compared with 4.5 mm. for the males.

Egg. The eggs are very small and difficult to find as they are laid principally in slits beneath the outer bark of plum trees and to a limited extent on peach (Fig. 1 C, D). The egg (Fig. 1 E) is pearly white, oval, elongate, slightly curved, tapered and rounded at both ends. Length 0.66 mm.

Nymph. The nymphs (Fig. 2) are reddish-brown, robust with broad abdomen lifted into a sharp crest, each segment of which ends in a tooth.

First instar. Robust body with short transverse head. Abdomen lifted into sharp crest, each segment ending in a tooth that projects posteriorly. Color reddish-brown, marked with fulvous vitta, fuscopunctate. Pronotum and scutellum fulvous. A broad fulvous vitta on the dorsal median line of the abdomen. A narrower fulvous vitta on either side of the abdomen beginning with the second segment and extending posteriorly to the lateral margin of the seventh and eighth tergites. Face and clypeus dark brown. Legs fulvous to dark brown. Abdomen salmon-colored beneath. Genital pieces infuscated. Length 2 mm.

Second instar. Color dark brown to black. Head, pronotum, and scutellum brown. A broad fulvous vitta contiguous to scutellum and a second

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[2] For technical description of adult see Breakey (3, p. 828–829).
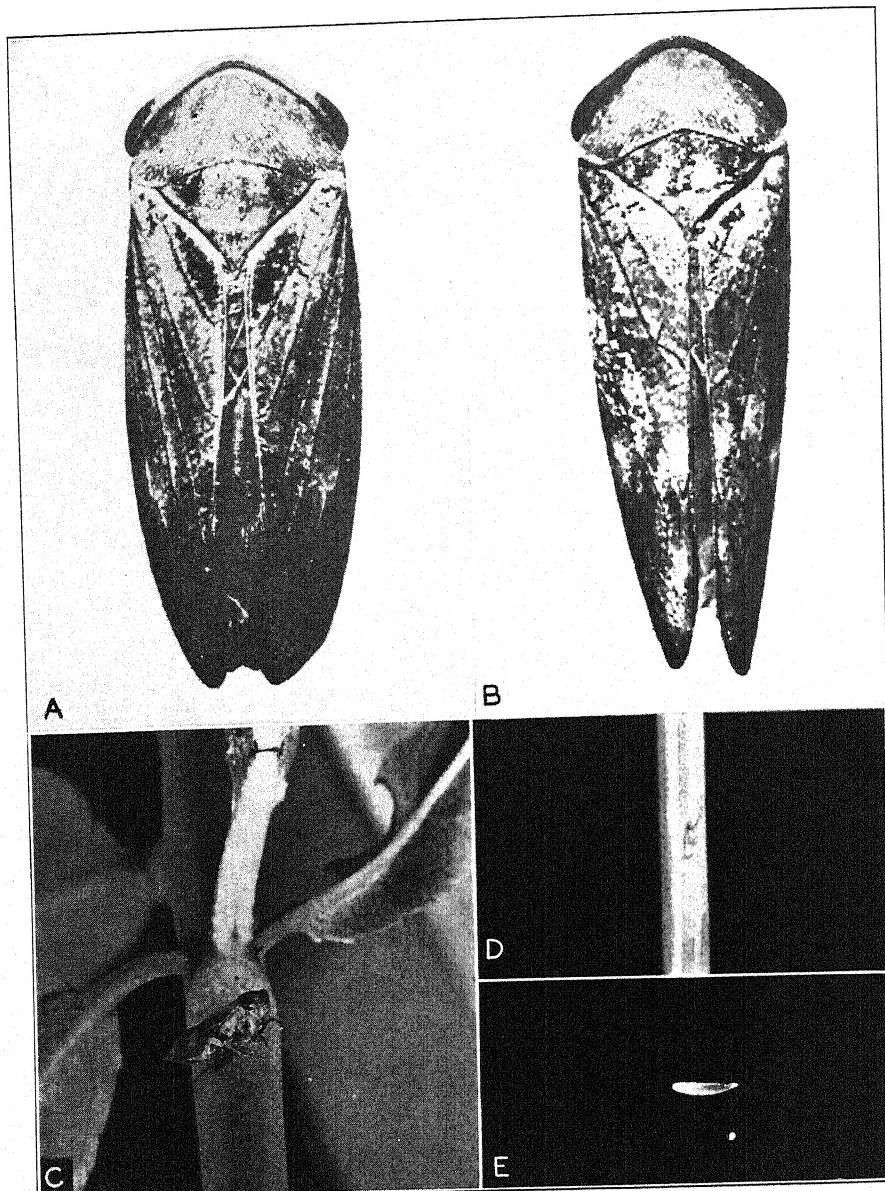
FIGURE 1. Plum and peach leafhopper, *Macropsis trimaculata*. (A) Adult female, ×18.
(B) Adult male, ×18. (C) Female ovipositing, ×3.5. (D) Egg scars, ×3.5. (E) Egg, ×7.
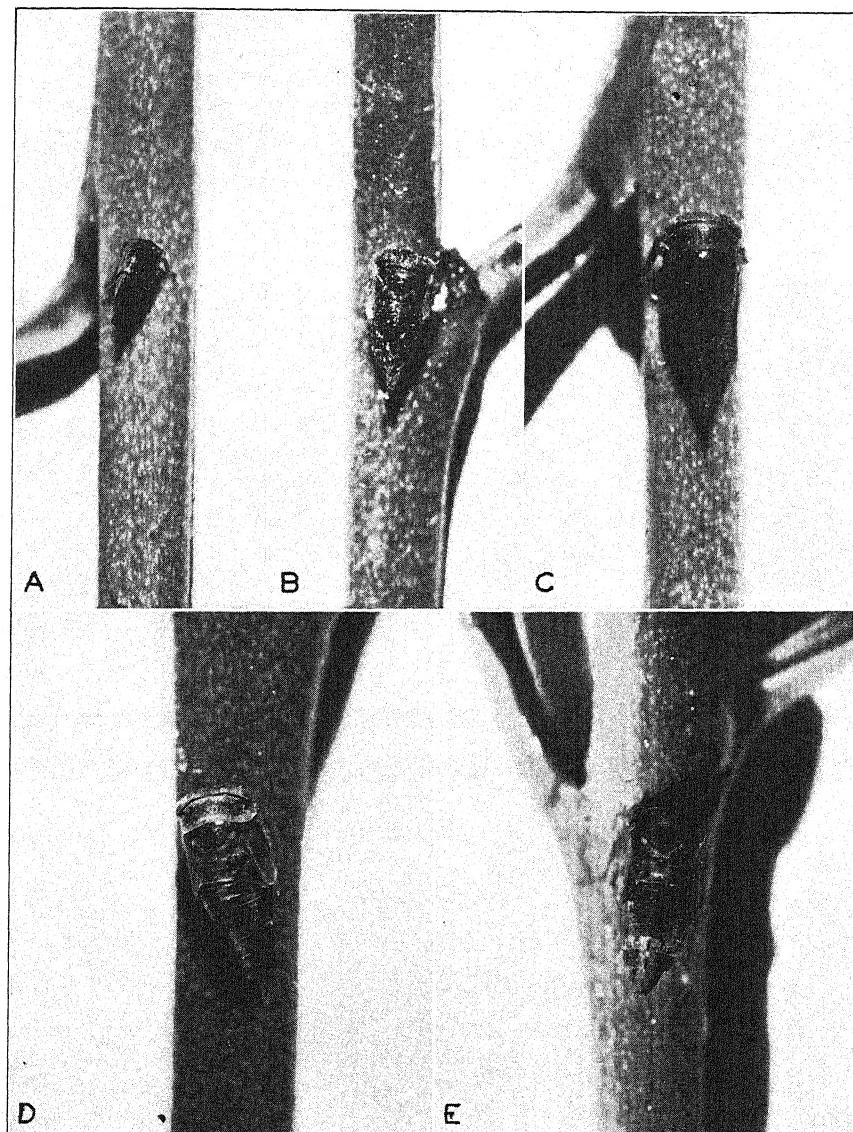
FIGURE 2. Nymphal stages of the plum and peach leafhopper, *Macropsis trimaculata*, ×7. (A) First instar. (B) Second instar. (C) Third instar. (D) Fourth instar. (E) Fifth instar.

just caudad to it. Abdomen dark brown to black, lighter beneath. Lateral markings more distinct than in first instar. Dorsal markings distinct in the sixth and seventh segments but less distinct or wanting in other segments. Face and clypeus dark brown. Legs yellow striped with black. Genital pieces dark brown to black. Length 2.75 mm.

Third instar. Body more robust than in the first and second instars. Color markings resembling second instar. Wing pads appear as lateral buds extending to the posterior margin of the first abdominal segment. Length 3 mm.

Fourth instar. General ground color black. Markings resembling second and third instars. Dorsal surface of head, pronotum and scutellum densely clothed with short gray hairs. Ventral surface of abdomen salmon-colored. Genital pieces fuscous. Legs yellow striped with black, pubescent. Wing pads extend to the posterior margin of the second abdominal segment. Length 3.5 mm.

Fifth instar. General body color reddish-brown. Eyes pink. Lateral markings of abdomen indistinct. Distinct white dorsal markings on the sixth and seventh tergites. Dorsal surface of head fulvous. Basal angles of scutellum black. Wing pads yellow, extending to middle of the third abdominal segment. Face dark brown. Clypeus and beneath yellow. Thoracic pleurites black. Legs yellow striped with black. Ventral surface of abdomen salmon-colored. Genital pieces infuscated. Seventh, eighth, and ninth tergites fulvous at crest with black tooth-like projections. Length 4 mm.

LIFE HISTORY

METHODS

In order to devise satisfactory methods for rearing this insect, considerable time was spent in perfecting means for keeping it under observation for a sufficient length of time to determine its bionomics. When this work was begun little was known of the life history of this species principally due to the fact that the insect had been overlooked because of its peculiar habits of concealment.

The most satisfactory method of collecting the nymphs was by brushing them from the twig with a moistened camel's hair brush into shell vials. Adults were collected (Fig. 3 A, B) and transferred by means of a pneumatic insect collector (13). This apparatus was found to be unsatisfactory for transferring the nymphs because of the difficulty experienced in dislodging them from the cheesecloth screen.

In order to determine the length of the nymphal instars, newly hatched nymphs were placed in glass vials (3 in. × 1/2 in.) containing twigs of peach or plum two inches in length. The leaf blades were removed, leaving the petioles intact. A nymph was placed in each vial. Examinations were made daily for molts and the exuviae removed. The twigs were replaced
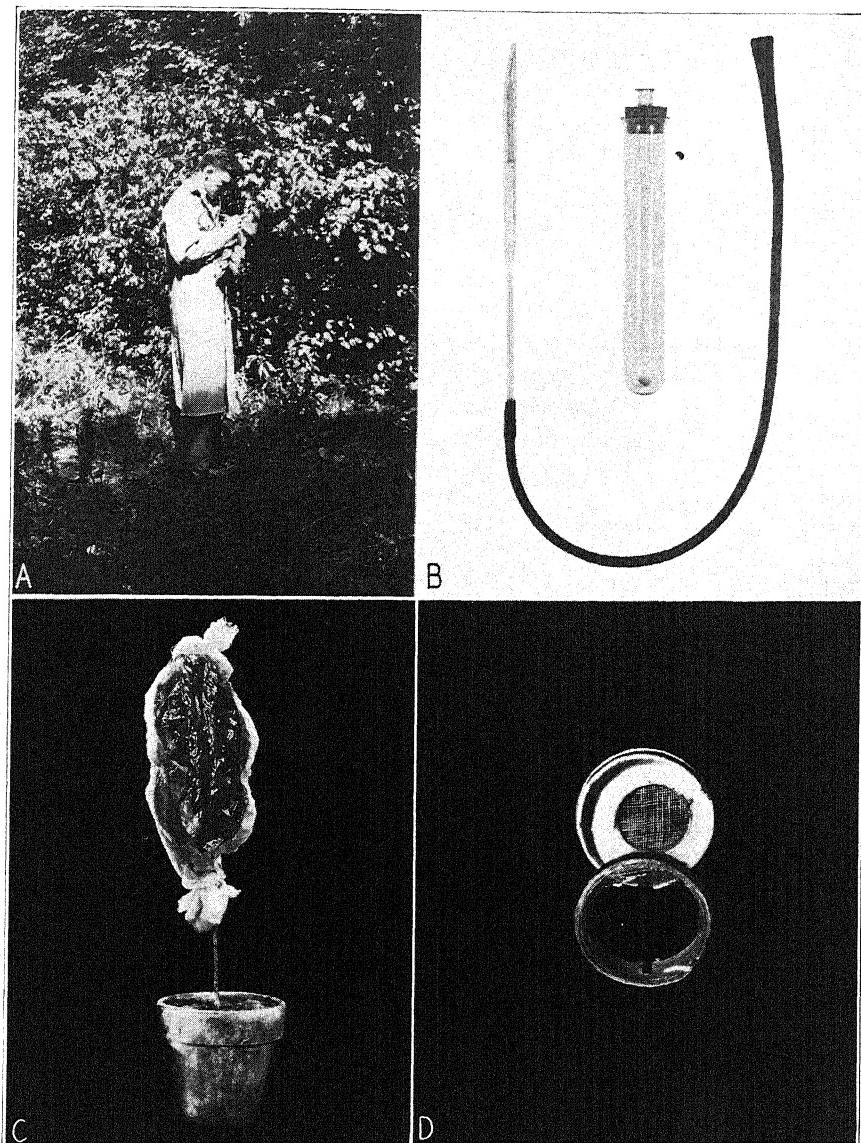
FIGURE 3. Apparatus and methods. (A) Collecting living adults of *Macropsis tri-maculata* by means of a pneumatic insect catcher, from wild plum trees. (B) Modified Kunkel insect catcher, with pipe-stem mouthpiece. In center, tube into which insects are transferred. (C) Cellophane-cheesecloth cage for enveloping small tree. (D) Cage for confining leafhoppers to a twig or a leaf-petiole. Made of pillbox with cellophane top and cheese-cloth bottom. Notches in rim to permit entrance of twig or leaf-petiole.

with fresh ones every other day. The vials were corked, inverted, and kept in a vertical position by placing them in a wire basket. Since the nymphs and adults are negatively geotropic they have a tendency to ascend the sides of the vial and wedge themselves between the cork and the glass sides. By inverting the tubes this difficulty is overcome. This method was used in making life history studies of the insect under constant temperature conditions. (For general description of temperature control see 30, p. 14.) Living adults and nymphs similarly placed in vials could be transported without injury by automobile for a distance of 75 miles or more.

For rearing insects in the greenhouse or in an out-of-door shelter, a number of different kinds of cages were used depending upon the object to be attained. In caging one-year-old plum or peach seedlings, lantern globe cages with the tops covered with fine wire screen (50 meshes per linear inch) were found satisfactory. The trees were in 4-inch pots resting on glass plates. When potted trees four or five years old were used, cages sufficiently large to accommodate two to four trees were employed. These cages were tightly constructed with wooden frames and floors. The sides were made of panes of glass while the ends and tops were covered with wire screen (50 meshes per linear inch). The greenhouse in which these cages were located was shaded by coating the glass with white paint. The temperature in the greenhouse ranged from 70° to 90° F. In the out-of-door shelter the temperature averaged about 10° F. lower during the growing season than the greenhouse temperatures.

Cages made by cementing together strips of cheesecloth and cellophane (Fig. 3 C) were employed in confining insects to branches or to the tops of small trees. For confining insects to individual twigs or leaves pill boxes with cellophane tops, cheesecloth bottoms, and with notches in the rims (Fig. 3 D) to permit the entrance of the twig or the leaf, were employed.

LONGEVITY OF ADULTS

The number of days the adults were kept in captivity ranged from 11 to 51 in the experiments under greenhouse conditions. The average for 34 observations was 25 days. The latest record was September 11.

The earliest date that adults were observed in the field at Yonkers, New York, was June 15, the latest was August 17. Adults were collected in the Transitional life zone in Connecticut as late as August 25. The ratio of males to females captured in June and the first week in July was 7:3 while for the latter part of the season the ratio was 5:9.

OVIPOSITION

The period of oviposition of this species is believed to be relatively long as indicated by the longevity of the adults and the time between the appearance of the first and last nymph. Fertile females were captured as

late as August 15. Females dissected on July 30 showed 20 to 25 developed eggs.

INCUBATION PERIOD

The winter is passed in the egg stage. Freshly-laid eggs fail to hatch until they have received a pre-treatment at low temperature. Under field conditions at Yonkers, New York, the egg period extends from the middle of July to May of the next year. On April 28, 1937, twigs of *Prunus americana*, *Prunus angustifolia* Marsh., and Red June plum from Delaware infested with eggs of *Macropsis trimaculata* were placed with the cut ends in water in the greenhouse at Yonkers, New York. Nymphs hatched from May 10 to 19 from eggs that had been deposited in these twigs. Twigs from this series placed in 12-inch test tubes in ovens at constant temperatures of 15°, 20°, and 25° C. failed to hatch any nymphs. Daily alternating temperatures of 15° C. for 16 hours and 20° or 25° C. for 8 hours also failed to hatch nymphs. The eggs also failed to hatch when twigs were placed on moist granulated peat moss. When twigs of *Prunus americana* from local trees infested with eggs were placed in flasks with the cut ends in water in ovens at constant temperatures of 15°, 20°, and 25° C., nymphs hatched on June 3. In the above experiments no attempt was made to control the humidity. In another experiment, however, no nymphs hatched from plum twigs infested with eggs kept constantly at room temperature in atmospheres of various relative humidities ranging from no moisture to about 90 per cent humidity.

The earliest dates that newly hatched nymphs of this species were observed in the field at Yonkers, New York, during the last five seasons are as follows: June 8, 1933, May 28, 1934, May 30, 1935, May 25, 1936, May 26, 1937. The last nymphs were collected in the field June 21, 1934, June 24, 1935, June 16, 1936, and June 23, 1937.

LENGTH OF NYMPHAL PERIOD

The duration of the nymphal period varies greatly depending upon temperature conditions. In an attempt to rear the nymphs at constant temperatures of 5°, 10°, 15°, 20°, and 25° C., the nymphal period was completed only at 20° C. At this temperature the nymphal period was greatly prolonged although the insect was able to pass through its five instars and become adult. Under field conditions the first instar varied from one to five days, the second instar from three to six days, the third instar from four to seven days, the fourth instar from six to eight days, and the fifth instar from seven to nine days. Thus the total nymphal period for 500 individuals observed under field conditions showed a minimum of 21 days and a maximum of 35 days. It will be noted in Table I that at a constant temperature of 20° C. the minimum period was 38 days while the maximum was 74 days with an average of 52.9 days. Under field conditions

the length in days of the nymphal instars is thus approximately one-half that given in Table I. Apparently a constant temperature at 20° C. while capable of supporting life is unfavorable for development. Alternating temperatures of greenhouse and field are more favorable for development. In this connection it is interesting to note a parallelism in these results with those obtained by Giersbach (8) in the germination of seeds of *Viburnum*. She found also that a temperature of 20° C. was the only constant temperature at which the seed would germinate although a low percentage germination was obtained, while optimum germination was obtained with alternating temperatures of 16 hours at 20° and 8 hours at 30° C.

Flemion and Hartzell (7) have called attention to the similarity in response to a period of low temperature in insects that hibernate in the egg stage and the germination of certain seeds of the colder temperate zone region that require a pre-treatment at low temperature before germination.

TABLE I

COMPARATIVE DURATION IN DAYS OF NYMPHAL INSTARS AT 20° C.

Stage	Minimum	Maximum	Average	Number of observations
1	1	14	4.3	117
2	3	16	9.6	17
3	11	14	12.5	2
4	11	12	11.5	2
5	12	18	15.0	3
Totals	38	74	52.9	141

TOTAL LIFE CYCLE

The egg period including dormancy during the winter months and incubation in the spring extends over a period of approximately ten months. The nymphal period averages about one month. The preoviposition period is believed to be comparatively long. While adults have appeared as early as June 15, they were not observed mating until July 13. With leafhoppers egg deposition does not begin until several days after mating. This would indicate a preoviposition period of about a month. Thus it requires 12 months to complete the cycle (Figure 4 B). To summarize, the overwintering eggs in the twigs hatch almost with the first growth of spring. The nymphs mature in June, while the females lay the eggs during July and August. The comparatively long time necessary for the species to reach maturity precludes the possibility of this insect being two-brooded under field conditions.

Ball (2) has called attention to the fact that species of leafhoppers that pass the winter in the egg stage develop earlier in the spring then do species that hibernate as adults. This he claims is a general adaptation found par-
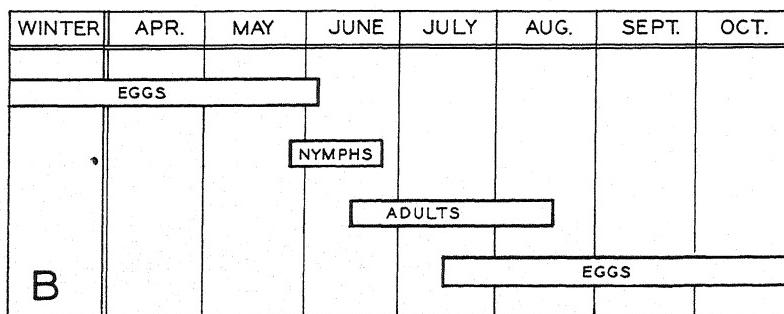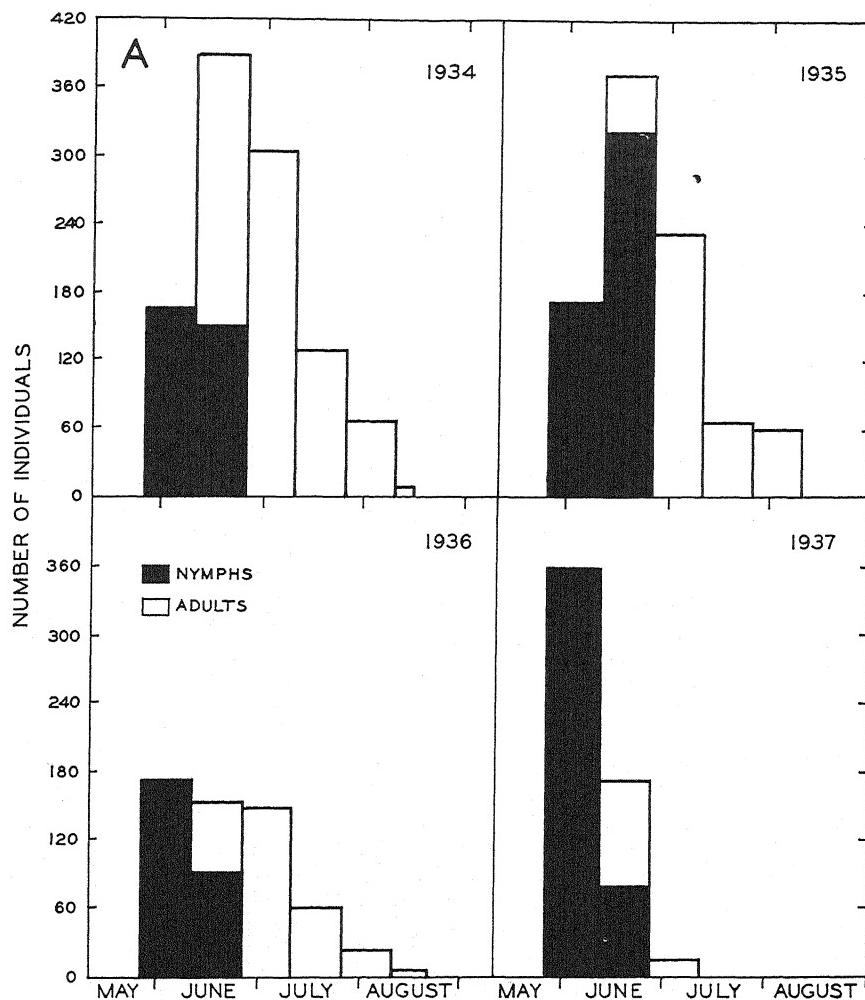
FIGURE 4. *Macropsis trimaculata*: (A) Population. (B) Life cycle.

ticularly in tree-infesting leafhoppers and is correlated with the short growth period in the spring. There is only one period of the year in which the female leafhopper has soft tender wood in which to deposit eggs and that is during the fore part of the growing season. The writer has observed females late in the season that had their ovipositors wedged into the wood so tightly that they had been held prisoners until they died. This observation would tend to confirm Ball's statement that species of leafhoppers that oviposit in twigs or branches of trees are necessarily restricted to a relatively short time for oviposition, and that the time that the wood is in the proper condition is too limited to permit more than one generation a year under natural conditions.

HABITS OF THE INSECT

FEEDING

In place of feeding on foliage as is the usual habit of leafhoppers, this species is found on the twigs and smaller branches and is rarely observed on the leaves. It was observed for the most part feeding on young twigs and only occasionally on the petiole and mid-rib of the leaves. Feeding is accomplished by puncturing the bark by means of slender stylet-like mandibles after which the maxillae are forced into the plant tissue and the sap extracted in the way characteristic of homopterous insects.

The insect is deleterious to young peach seedlings when exposed in large numbers. Kunkel (14) reports that if as many as ten adults are allowed to feed on a young peach seedling one foot high for a period of one week, they cause a sudden wilting of the tree, which may result in the death of the tree unless the insects are promptly removed. If this is done in time the tree recovers. As he was unable to find any bacteria or fungi associated with the tissue from wilted trees, he suggests that the insect may inject a deleterious substance that in some way causes the wilting.

HABITS OF THE ADULTS

The adults run rapidly and seek concealment on the opposite side of the limb from the observer. This combined with their color which resembles the bark of the trees makes them difficult to collect. They very seldom take flight when approached, as is the usual habit of leafhoppers, but are capable of a rapid zig-zag flight when moving from tree to tree or during mating activity. They seek the shade during the hottest part of the day. Males and females were observed mating on cultivated plum July 13 and 19, 1937.

HABITS OF THE NYMPHS

It was observed that the greatest number of nymphs hatched in the morning. During May, 1937, this period came between 8:30 and 10 A.M.

E. S. T. A similar periodicity was reported by the writer (5) for *Empoasca fabae* Harris.

The newly emerged nymphs are found concealed between the unfolding leaves. Later they are found on the twigs and small branches. They run rapidly and seek concealment on the opposite side of the limb from the observer, closely resemble the bark, and are easily overlooked. They rarely hop although physically able to do so if probed.

SEASONAL HISTORY

HIBERNATION

There appears to be a very close relationship between the dormancy of the egg and the dormancy of the twig in which it is laid. That the period is not merely a delay in hatching without relation to temperature is shown by the fact that eggs deposited on plum trees and kept in the greenhouse all winter failed to hatch. Plum trees infested with eggs were placed in a cold room in September at a constant temperature of 10° C. for a period of two months after which they were returned to a warm greenhouse. Nymphs hatched from the eggs deposited in these trees during the last week of November.

No evidence of a second generation was observed under field conditions during the past five seasons that the species was under observation.

POPULATION STUDIES

Nymphs and adults of *Macropsis trimaculata* were collected over a three-hour period daily, weather permitting, throughout the growing season. Most of the captures were from wild plum but some were also taken from domesticated plum and peach. Collections were made at several localities in Connecticut, New York, New Jersey, and Pennsylvania, but only insects collected at Yonkers, New York, are included in the population studies.

In 1934 the total number of nymphs collected was 166 during the 15-day period from May 28 when the first nymphs were observed in the field to June 11. The insect reached its maximum population in the period from June 12 to June 26 when a total of 389 nymphs and adults were captured. It then declined very rapidly as indicated in Figure 4 A, but adults could be found sparingly until the middle of August. The population was the largest for the five-year period studied. This was in spite of the fact that the eggs during the dormant period were exposed to unusually low temperatures. The thermometer dropped to −6° F. during December, 1933, at the nearest local weather station (22). In 1935 the number of nymphs collected was 173 during the 15-day period from May 30 to June 13. The maximum insect population was reached during the following 15-day period with a total of 372 nymphs and adults captured. The decline

then was even more rapid than in 1934. The last adults were collected August 9.

The season of 1936 was unfavorable for leafhopper development due no doubt to an unusually heavy precipitation during June (22). The total depth of water for June 1936 was 4.66 inches as compared to 3.46 for the 68-year average of the nearest local weather station. The heavy rainfall in the fore part of the season no doubt led to the destruction of a large number of newly emerged nymphs. From May 25 to June 8, 170 nymphs were collected. The total insect population fell off very rapidly during June and July. The last adults were captured on August 17.

The season of 1937 was somewhat more favorable for leafhopper development than the previous season. During the first 15-day period from May 26 to June 9, 362 nymphs were captured. The number of adults taken, however, was very small and confined to a short season, as the last adults were captured July 9.

Compared with studies conducted by Manns (21) who estimated in one instance the population of a plum tree in Delaware at 10,000 the population of *Macropsis trimaculata* at Yonkers, New York, is sparse. The fact that our captures were confined largely to *Prunus americana* in a suburban area may account for this. The writer has found, for example, much larger populations on cultivated plum at Southbury, Connecticut than at Yonkers, New York. As it was not found feasible to make daily collections at stations far removed from Yonkers, the data as presented show the relative distribution in population throughout the season, without regard to the maximum and minimum numbers for the whole range of the species.

SUMMARY

The plum and peach leafhopper, *Macropsis trimaculata* (Fitch), has been reported only from North America. In the United States it occurs as far west as Colorado, and south as far as Virginia. Its northern range includes southern Ontario and Quebec, Canada.

The principal wild host of the plum and peach leafhopper is *Prunus americana* but it also occurs on *Prunus angustifolia*, *Prunus munsoniana*, and *Prunus pissardi*. It occurs sparingly on peach and in greater numbers on *Prunus domestica* but prefers Japanese and Chinese plums.

Macropsis trimaculata, as the vector of peach yellows and little peach, causes heavy losses to both peach and plum plantings throughout the area in which these diseases are endemic. The injury to plum has been until recently overlooked because on plum, yellows is masked.

Under natural conditions there is only one brood of *Macropsis trimaculata* each year. The insect passes the winter in the egg stage in slits beneath the outer bark of plum and to a lesser extent in peach. The eggs hatch

during the latter part of May and the early part of June in the latitude of Yonkers, New York.

The nymphal period requires from 21 to 35 days under field conditions. Nymphs reared at constant temperatures of 5°, 10°, 15°, 20°, and 25° C. were able to complete their development and become adults only at 20° C. The nymphal period, however, was greatly lengthened at this temperature, with a minimum of 38 days and a maximum of 74 days.

Adults emerged under natural conditions from the middle of June until the first week in July. They were collected in the field as late as the middle of August.

Unlike most species of leafhoppers, both adults and nymphs feed on the twigs and are only occasionally observed on the foliage. They run rapidly over the twigs and the adults do not fly when approached nor do the nymphs hop as is the usual habit of leafhoppers, but seek concealment in the forks of the limbs or on the opposite side of the branch from the observer.

The population distribution is also erratic depending upon host preference, rainfall at the time the eggs hatch, and on other ecological factors.

Aside from a species of spider that was found feeding on a nymph, no predacious or parasitic enemies of *Macropsis trimaculata* were observed.

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CARBON DIOXIDE STORAGE. X. THE EFFECT OF CARBON DIOXIDE ON THE ASCORBIC ACID CONTENT, RESPIRATION, AND pH OF ASPARAGUS TISSUE[1]

NORWOOD C. THORNTON

The economic importance of supplying the consumer with fresh vegetables justifies the use of any practical method that will keep the vegetables in good condition during transportation from the producer. Rapid refrigeration of freshly-harvested vegetables soon after harvest and during transportation has been the most practical way of preserving quality and freshness. For this purpose water ice is commonly used. However, in recent years there has been a demand for a more rapid cooling of the vegetables after loading into refrigerator cars, for which solid carbon dioxide has been used. Besides its effectiveness as a refrigerant in the solid state, the carbon dioxide gas accumulating in the atmosphere is also beneficial in other respects, as it may retard respiration, preserve the freshly-harvested appearance, and retard the growth of fungi that would impair the quality of the vegetables during shipment. Brooks, Bratley, & McColloch (1) have found this to be true for many vegetables, especially asparagus. However, from previous experience of the writer there arose the question of the effect of the carbon dioxide on the desired food quality of the vegetables. As reported (10) the storage of potatoes in carbon dioxide is not desirable because of the more rapid breakdown of starch to sugar which is associated with the very great increase in the rate of respiration of potatoes under this treatment. However, the effect of carbon dioxide on the rate of respiration of asparagus is just the reverse of that found with potatoes. Since many workers (2, 6, 7, 11, 12) have shown that low temperature storage, which retards the rate of respiration, will also effectively retard the loss of the ascorbic acid (vitamin C) content of vegetables, one may postulate that an exposure of asparagus to carbon dioxide would retard the loss of its ascorbic acid content. However, the experimental work shows that although carbon dioxide does retard the rate of respiration of asparagus tissue, it will also cause a rapid loss of the ascorbic acid content of the tissue.

MATERIAL AND METHODS

Asparagus (*Asparagus officinalis* L.) shoots cut and bunched in the usual commercial manner were obtained from a farm in Maryland. When the experiments were started all of the asparagus was in excellent condition

[1] Presented before the Division of Biological Chemistry, Symposium on Vitamins, at the 94th meeting of the American Chemical Society, Rochester, New York, September 7, 1937.

as it had been kept moist without refrigeration and was not over 20 hours old, i.e., from time of cutting. For a few of the experiments (designated in the text) the asparagus was obtained through a local wholesale dealer and it was about 36 hours old. Carbon dioxide injury to asparagus previously reported (8, p. 234) did not occur in these experiments because of the freshness of the material. The previous results were repeated and verified, however, with asparagus 48 to 72 hours old before subjection to the treatment with carbon dioxide. The difference in the age of the experimental material at the start of the treatment with carbon dioxide will account for the apparent differences between the previous work (8) and that reported elsewhere (1). Before setting up the experiment each bunch of asparagus was cut so that every shoot would be 15 cm. long from tip to base and was placed in water for 10 minutes. The shoots were then so graded that approximately 2 grams of bud tissue would be obtainable. The gross weight of the shoots placed in every condition of the experiment was found at the beginning and at the end of each test and the results indicate that there was never more than 1 gram difference in the loss of weight of the control and of the lot receiving the highest carbon dioxide treatment during storage. The total weight loss during each experimental period varied from 2 to 5 grams for 175 to 400 grams of asparagus tissue. From 30 to 60 shoots were used in each test condition and from this number duplicate lots of 10 stalks each were used for each determination of ascorbic acid content and pH.

The storage rooms at various constant temperatures, the 8-liter tin cans for storing the asparagus under treatment, the apparatus for determining rate of respiration, and the method for obtaining the desired gas mixtures in the atmosphere about the asparagus, have been discussed in a previous report (9). In every treatment 20 per cent of oxygen was used and the carbon dioxide and nitrogen content of the gas mixture was varied according to the treatment. The three gases used to make up the artificial atmospheres were obtained from cylinders of the compressed gases. In some cases the asparagus tissue was held in an atmosphere relatively free of respired carbon dioxide. This atmosphere was obtained by placing in the can a beaker containing a paper moistened with 4 per cent sodium hydroxide. A small opening was left in the can to allow air to enter to avoid a vacuum being produced during the test. Furthermore, a sample of the air in the can was analyzed for carbon dioxide at the end of each experiment and the maximum amount ever found was 0.3 per cent.

The asparagus tissue, when removed from the storage conditions, was washed with distilled water and the excess moisture removed with cheesecloth. A sample of the tissue was ground through a food chopper, and the juice extracted by squeezing by hand through a double thickness of cheese-

cloth. The pH of the juice was then determined with the quinhydrone electrode, using a saturated calomel half cell as reference electrode.

The ascorbic acid content of the asparagus bud and stalk tissue was determined as follows: A weighed quantity (approximately 20 grams) of tissue was ground by the aid of acid washed quartz sand to an extremely fine consistency in 50 cc. of a mixture of acids consisting of 5 per cent sulphuric and 2 per cent metaphosphoric as suggested by Mack and Tressler (5). This preparation was then transferred to a 200 cc. volumetric flask, made up to volume with the acid mixture, thoroughly mixed, centrifuged and aliquots used for titration with a solution of 2,6-dichloro-phenolindophenol. The indicator solution was prepared each day and was standardized against a freshly prepared solution of ascorbic acid in the acid mixture.

EXPERIMENTAL RESULTS

The effectiveness of carbon dioxide in bringing about changes in ascorbic acid content, rate of respiration, and pH of the asparagus tissue during storage of 24 hours at 22° C. is shown in Figure 1. During this period the
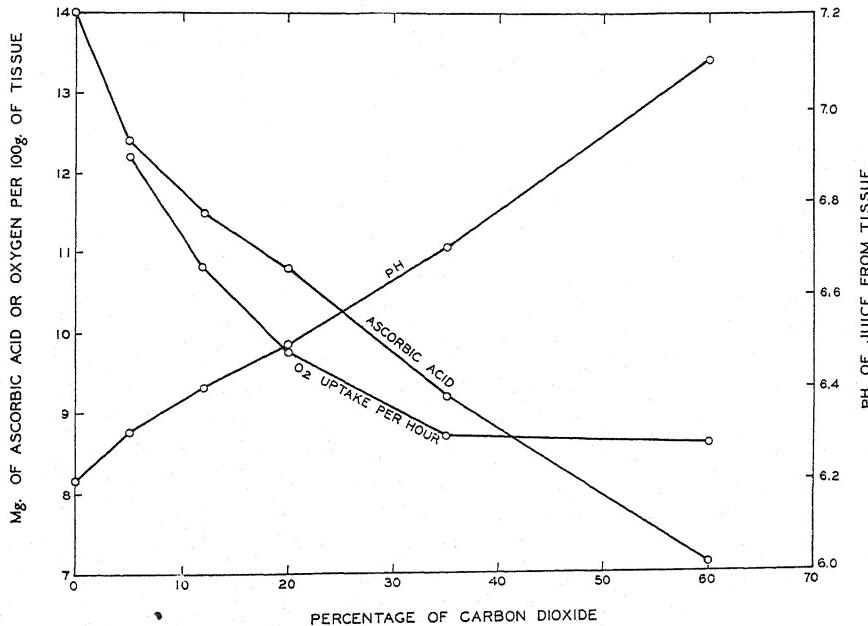
FIGURE 1. The effect of carbon dioxide on the ascorbic acid content, rate of respiration, and pH of asparagus tissue during storage of 24 hours at 22° C.

asparagus that originally contained 19.0 mg. of ascorbic acid per 100 g. of bud tissue lost 5 mg. when held in air relatively free of carbon dioxide

and 11.9 mg. when held in 60 per cent of carbon dioxide. This means an actual loss of 6.9 mg. of ascorbic acid that may be attributed directly to the effect of the treatment with carbon dioxide. On a percentage basis the control sample of asparagus lost 26.3 per cent and the carbon dioxide treated sample 62.6 per cent of the original ascorbic acid content during the 24 hours at 22° C. or a difference of 36.3 per cent that may be attributed directly to the effect of the carbon dioxide treatment. From this we may estimate that the loss in ascorbic acid content of asparagus treated with carbon dioxide for 24 hours at 22° C. is equivalent to the loss occurring in approximately 58 hours of storage in ordinary air at this temperature. Intermediate concentrations of carbon dioxide likewise caused a loss in ascorbic acid content of the bud tissue as shown in Figure 1. Furthermore, the accumulation of carbon dioxide from respiration is also detrimental to asparagus during storage. As shown in Figure 1, 5 per cent of carbon dioxide accumulating in the closed container (without NaOH) during the test brought about a total loss of 34.7 per cent of the ascorbic acid content of the tissue which is 8.4 per cent greater than the loss in the control tissue held in the absence of carbon dioxide. This result is of particular significance when one considers that vegetables are sometimes held for various periods in relatively air-tight and closely packed storage rooms where carbon dioxide may accumulate from the respiration of various plant materials.

The effectiveness of carbon dioxide in reducing the rate of respiration (measured as mg. oxygen uptake per 100 g. of tissue per hr.) of the asparagus tissue is also shown in Figure 1. The presence of 12 per cent of carbon dioxide reduced the rate of respiration 15 per cent, and 35 per cent CO_2 reduced it 29 per cent below that of the tissue held as control. Of course the control tissue in this case was exposed to accumulating carbon dioxide so that its rate of respiration would be continuously lowered; thus we may suggest (unpublished data) that the reduction due to carbon dioxide was even greater than that shown in Figure 1.

Concurrent with the loss in ascorbic acid content and reduction in rate of respiration there occurred a very great increase in the pH of the asparagus tissue treated with carbon dioxide. As shown in Figure 1, the tissue changes from pH 6.2 in the absence of carbon dioxide to pH 7.1 in the presence of 60 per cent of carbon dioxide. Even in this case a small quantity of respired carbon dioxide was effective in bringing about a measurable increase in the pH of the tissue.

In view of the fact that ascorbic acid may be oxidized to dehydroascorbic acid which is not detected in the titration with indophenol, care was taken to insure that the ascorbic acid in the extract was in the reduced form. The asparagus extract was treated with hydrogen sulphide for 15 minutes and the excess H_2S was eliminated by bubbling carbon dioxide or nitrogen through the extract. The ascorbic acid content of the asparagus

extract before and after the H_2S treatment was found to be the same as is shown by the data in Table I. Since ascorbic acid has not been found in intact vegetables in any other than the reduced form, these data bear out the fact that the carbon dioxide treatment has brought about a partial destruction of the ascorbic acid content of the asparagus tissue. Whether the ascorbic acid is rapidly oxidized to dehydroascorbic acid and with further decomposition is lost as far as its measurement is concerned or whether it is destroyed by some other method in the tissue during the treatment with carbon dioxide is at present unknown. Until further evi-

TABLE I

EFFECT OF H_2S REDUCTION OF ASPARAGUS EXTRACT IMMEDIATELY AFTER GRINDING ON ITS
ASCORBIC ACID CONTENT

Treatment of intact tissue	H_2S reduction of asparagus extract before titration	Ascorbic acid mg. per 100 g. of tissue		
		Intact tissue held in CO_2 treatment at:		
		10° C.	17° C.	20° C.
Control in closed container	No	15.2	15.3	10.5
	Yes	15.1	15.3	10.5
10% CO_2	No	14.6	12.5	9.9
	Yes	14.5	12.3	9.9
60% CO_2	No	11.3	8.1	7.3
	Yes	11.3	8.1	7.3

dence is obtained we may consider the differences found in ascorbic acid content of treated and untreated tissues as the result of its destruction by some agency which is acted upon by the carbon dioxide.

Having found that carbon dioxide was effective in causing the loss of the ascorbic acid content of the asparagus tissue, the next problem was to determine if the time and temperature of treatment would alter this result. The data in Table II show that only a short period of storage in carbon dioxide is necessary to cause the greater proportion of the loss in ascorbic acid. The ascorbic acid content of asparagus held for five hours at 27° C. in the closed container had decreased from 20.5 mg. at the start to 16.9 mg. or a loss of 17.5 per cent due to the combined effect of temperature and accumulation of carbon dioxide from respiration. At the same time the asparagus exposed to 30 and to 60 per cent of carbon dioxide lost 35.6 and 48.2 per cent respectively of its original ascorbic acid content. During the total period of 26 hours of storage the asparagus held as control lost 32.7 per cent of its original ascorbic acid content while the asparagus exposed to 30 and to 60 per cent of carbon dioxide lost 57.1 and 52.7 per cent

respectively. From this information we see that the greater proportion of the total loss of ascorbic acid took place during the first five hours of storage in carbon dioxide.

As the temperature of storage was lowered to 20° C. an entirely different lot of asparagus was used (asparagus 36 hours old from wholesaler's refrigeration room) which contained 15 mg. of ascorbic acid per 100 grams of bud tissue. During the 22 hours of storage the tissue held as control in the closed container lost 31.3 per cent of its original ascorbic acid content and during the next 22 hours of storage it lost an additional 16 per cent. In the same experiment the decrease in ascorbic acid content of the asparagus held in 30 and 60 per cent of carbon dioxide for 22 hours was 58.6 and

TABLE II

EFFECT OF DURATION OF CARBON DIOXIDE TREATMENT AND TEMPERATURE OF STORAGE ON ASCORBIC ACID AND pH OF ASPARAGUS TISSUE

Treatment	Determinations	Hours of storage at temperatures of:						
		27° C.		20° C.		10° C.		
		5	26	22	44	45	67	89
Control in closed container	Ascorbic acid*	16.9	13.8	10.3	7.9	15.3	13.3	11.7
	pH**	6.3	6.3	6.2	6.2	6.3	6.3	6.3
30% CO₂	Ascorbic acid*	13.2	8.8	6.2	5.5	8.6	8.4	7.2
	pH**	6.6	6.5	6.6	6.6	6.8	6.7	6.7
60% CO₂	Ascorbic acid*	10.6	9.7	6.8	5.0	8.9	8.1	5.7
	pH**	6.7	6.7	6.7	6.6	7.0	6.9	6.9

* Milligrams per 100 grams of bud tissue.
** Bud and stalk about 15 cm. long ground and juice extracted for determinations.

54.6 per cent respectively, and with another 22 hours of storage the asparagus lost only an additional 4.7 and 12.0 per cent respectively. In another experiment conducted at 10° C. with asparagus having an original concentration of 21.1 mg. of ascorbic acid the results show, in Table II, that 60 per cent of carbon dioxide reduced the ascorbic acid content 57.8 per cent in 45 hours and only an additional 15.2 per cent with another 44 hours of storage. This information leads to the conclusion that short periods of storage of asparagus in carbon dioxide are proportionately more detrimental than extended periods.

Furthermore, the data in Table II show the effectiveness of lower temperatures in retaining the ascorbic acid content of asparagus tissue. The asparagus held for 26 hours at 27° C. lost a greater percentage of its ascorbic acid content than the asparagus held for 45 hours at 10° C.

The effect of the duration of the carbon dioxide treatment at various temperatures on the pH of the asparagus tissue is also shown in Table II. Regardless of the temperature or the duration of the treatment there is always an increase in pH (decrease in hydrogen-ion concentration) of the tissue. Apparently the changes that take place in the tissue treated with carbon dioxide are exceedingly rapid to allow for such an enormous decrease in the H-ion concentration as represented by an increase of 0.4 of a pH unit in such a short period of treatment as 5 hours. In fact, the change is so rapid that a definite increase in pH of the asparagus tissue may be observed with only a 20-minute treatment with carbon dioxide.

The results of a further investigation of the combined effect of the storage temperature and carbon dioxide treatment on the ascorbic acid content of asparagus are given in Table III. These tests were conducted

TABLE III

STORAGE TEMPERATURE AND ACTION OF CARBON DIOXIDE ON ASCORBIC ACID AND pH OF ASPARAGUS TISSUE

Treatment	Asparagus under treatment					
	24 hrs. at 2° C.		28 hrs. at 17° C.		26 hrs. at 27° C.	
	Ascorbic acid mg./100 g. bud	pH bud & stalk	Ascorbic acid mg./100 g. bud	pH bud & stalk	Ascorbic acid mg./100 g. bud	pH bud & stalk
Control in room	18.7	6.0	18.4	6.2	18.6	6.2
Control in closed container	14.8	6.3	12.3	6.3	13.8	6.3
30% CO₂	13.2	6.6	9.2	6.4	8.8	6.5
60% CO₂	16.7	6.7	9.2	6.6	9.7	6.7

at the same time with fresh asparagus of the same age. In general the previously discussed effect of the carbon dioxide on the asparagus tissue is observed. The tissue held in air relatively free of carbon dioxide maintains a high concentration of ascorbic acid while the tissue exposed to either a low concentration of carbon dioxide from respiration or a high concentration in treatment loses a large proportion of its ascorbic acid content either at 2° or 27° C. Also the carbon dioxide is effective in increasing the pH of the asparagus tissue at any of the temperatures of storage.

Although at temperatures of 17°, 22°, and 27° C. the effect of carbon dioxide is to bring about a loss of ascorbic acid during treatment, the situation is quite different at a temperature of 2° C. and probably also at 10° C. At such temperatures the effect of 60 per cent of carbon dioxide is to retard the loss of ascorbic acid during the period of treatment. Thus, in Table III, column 2 at 2° C., it is seen that the ascorbic acid content of the

sample treated with 60 per cent carbon dioxide is definitely higher than that of the control sample held in the container or of the sample held in 30 per cent of carbon dioxide. Similarly, in Table IV, at 10° C. the sample

TABLE IV

CHANGES IN ASCORBIC ACID CONTENT AND pH OF ASPARAGUS TISSUE AS A RESULT OF EXPOSURE TO CARBON DIOXIDE AND EFFECT OF THIS TREATMENT ON SUBSEQUENT CHANGES WHEN HELD IN CO_2-FREE AIR

Treatment	After treatment for 27 hrs. at 10° C.		In CO_2-free air* for 24 hrs. at 10° C. following CO_2 treatment	
	Ascorbic acid mg. per 100 g. of bud tissue	pH of bud and stalk	Ascorbic acid mg. per 100 g. of bud tissue	pH of bud and stalk
Control in closed container*	15.4	6.1	14.2	6.1
Control in closed container	12.7	6.3	12.6	6.1
30% CO_2	10.0	6.9	10.0	6.1
60% CO_2	13.0	7.0	10.6	6.2

* CO_2 continuously removed with paper moistened with 4 per cent NaOH.

treated with 60 per cent carbon dioxide is higher in ascorbic acid than the sample treated with 30 per cent carbon dioxide and approximately equal to the control under the same conditions. However, when the tissue is removed to air relatively free of carbon dioxide the ascorbic acid content of the asparagus previously treated with 60 per cent of carbon dioxide decreased very rapidly to a substantially low value as compared with the other treatments. The investigation to date has not yet disclosed the exact nature of the combined effect of the high concentration of carbon dioxide with low temperature on the ascorbic acid content of asparagus tissue.

Heretofore there appeared to be some correlation between the increase in pH and the loss of ascorbic acid, but the data in Tables III and IV do not completely substantiate this idea. In both cases there are data to show that the ascorbic acid content of the asparagus tissue was not reduced to any great extent, although the pH of the tissue was greatly increased with the carbon dioxide treatment. Furthermore, in Table IV the asparagus upon removal to air after treatment with 60 per cent of carbon dioxide lost 18.4 per cent of the ascorbic acid content it contained at the end of the treatment and at the same time the pH of the tissue dropped from 7.0 to 6.2. These results indicate that the changes in asparagus tissue are the effect of the treatment with carbon dioxide on the metabolism of the tissue and not the direct action of the gas on ascorbic acid. In fact, carbon dioxide gas bubbled through freshly extracted asparagus juice has no effect on its ascorbic acid content; nor does the storage of the asparagus tissue

in high oxygen concentration bring about any more rapid destruction of the ascorbic acid than storage in a normal atmosphere.

The data in Table IV also show the effect of holding the asparagus tissue in an atmosphere relatively free of carbon dioxide following a period of treatment with the gas. During the treatment for 27 hours at 10° C. the tissue lost some of its ascorbic acid content and became considerably more alkaline. Following the treatment the tissue held in air relatively free of carbon dioxide for 24 hours at 10° C. changed very little in ascorbic acid content (except as already discussed) and the pH of the tissue returned to the original level. However, the ascorbic acid content of the tissue treated with carbon dioxide was still lower than that of the untreated tissue.

The ascorbic acid content of the asparagus stalk was reduced during the period of treatment with carbon dioxide as is shown in Figure 2. In
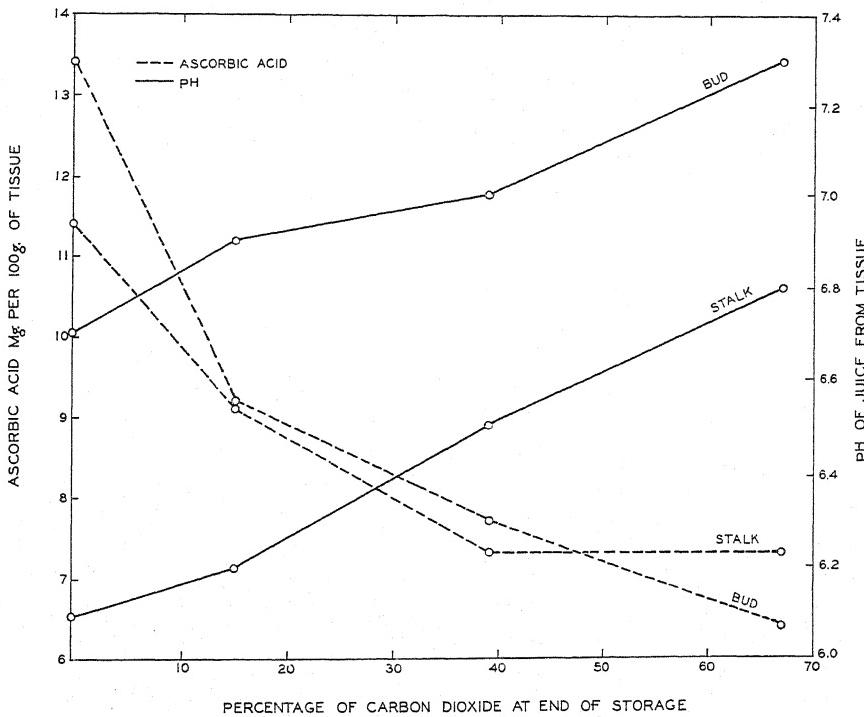
FIGURE 2. The effect of carbon dioxide on the ascorbic acid and pH of the asparagus bud and stalk tissue as determined after treatment for 42 hours at 22° C.

this test the intact asparagus shoot was held under the various conditions of storage for 42 hours at 22° C. and then removed and the bud taken in one sample and 4 cm. of the stalk immediately below the bud used in the other sample of tissue for analyses. The actual difference in ascorbic acid

content of the stalk held as control (in a container with NaOH) and that treated with 60 per cent of carbon dioxide was 4.1 mg. or 36 per cent of that contained in the control tissue. During this test, with the asparagus held in the closed container where the carbon dioxide was allowed to accumulate to a concentration of 15 per cent by volume, the ascorbic acid content of the stalk tissue decreased 2.3 mg. which is a loss of 20.2 per cent of that contained in the control tissue. At the same time the bud tissue exposed to 15 per cent of carbon dioxide (in the closed container) lost 31.3 per cent and that exposed to 67 per cent of carbon dioxide lost 52.2 per cent of the ascorbic acid content as compared with the tissue held in the absence of carbon dioxide. From these results it is apparent that the decrease in ascorbic acid is not a result of a movement of the ascorbic acid from the bud into the stalk but that there is an actual destruction of it in the tissue during the treatment with carbon dioxide.

The effect of carbon dioxide on the pH of the asparagus bud and stalk tissue is also shown in Figure 2. The bud tissue is more alkaline than the stalk immediately below the bud. However, the increase in pH of the bud from pH 6.7 to 7.3 and the stalk from pH 6.1 to 6.8 with a treatment of 60 per cent of carbon dioxide for 42 hours is in agreement with the results already discussed.

DISCUSSION

The results presented in this paper show that the accumulation of carbon dioxide in the air surrounding asparagus tissue causes a loss in the vitamin C content of the tissue. This means that any method of handling asparagus which allows for the accumulation of carbon dioxide about the tissue either from respiration or from the use of solid carbon dioxide as a pre-cooling agent is not desirable from the standpoint of preserving a high vitamin C content. However, if the appearance of the asparagus is of more importance than its vitamin content, then it is desirable to handle it in an atmosphere containing some carbon dioxide. Since the temperature of storage and length of exposure to carbon dioxide can not be varied to offset the destructive action on the ascorbic acid content, the only recourse at present is to insure that the asparagus is not exposed to a high concentration of carbon dioxide. A further investigation of the effect of carbon dioxide on the ascorbic acid content of other plant tissues is being conducted and the results will be reported at a future time.

Fellers and Stepat (3) and Fellers, Stepat, & Fitzgerald (4) have reported considerable loss of the vitamin C content of peas and lima beans in the pods during shipment even though the material was packed with ice. Possibly respired carbon dioxide is the detrimental factor in these cases since it could accumulate to a high concentration in the pods especially when the pods are packed closely together in the hampers. Tressler, Mack, and Jenkins (11) have reported a more rapid loss of ascorbic acid

from shelled lima beans than from unshelled beans. This no doubt is the result of the increased rate of respiration of shelled beans over unshelled beans (9) together with the resulting faster accumulation of carbon dioxide.

Attention is again directed to Figure 1, where is shown the effect of carbon dioxide on three interdependent measurements made on asparagus tissue. Theoretically only the depressing effect on the rate of respiration is to be expected. However, ascorbic acid is destroyed although the oxidation processes in the system are retarded. It is probable that the accumulation of end products from some associated metabolic processes, as evidenced by the increase in pH of the tissue, bring about the destruction of ascorbic acid without the aid of oxygen from the external atmosphere or by some process not yet investigated. These results were determined entirely by the titration method which is generally accepted as giving comparable results with the animal bioassay method.

SUMMARY

The ascorbic acid, vitamin C, content of asparagus tissue was reduced by 8 to 52 per cent with storage in an atmosphere containing carbon dioxide either added purposely or allowed to accumulate during respiration.

The loss of ascorbic acid was most rapid during the early hours of exposure to carbon dioxide and it occurred either during or following storage at all temperatures from 2° to 27° C.

The loss of ascorbic acid took place to the greatest extent in the bud tissue but it also occurred in the stalk.

After the loss of ascorbic acid during storage in carbon dioxide, there was no recovery when the tissue was exposed to the air after treatment.

The rate of respiration of the asparagus tissue was reduced during exposure to the carbon dioxide.

The hydrogen-ion concentration of asparagus tissue was decreased during the period of treatment with carbon dioxide. This was shown by an increase of 0.4 to 0.9 of a pH during treatment. The acidity returned to the original values upon removal of tissue to air.

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THE WEATHERING OF BORDEAUX MIXTURE[1]

FRANK WILCOXON AND S. E. A. McCALLAN

In a previous paper (6) it was shown that fungous spores excrete substances which are able to dissolve copper from sprayed films of Bordeaux mixture. These findings lend considerable support to the hypothesis that such spore excretions are responsible for the fungicidal action which is observed even when spore germination experiments are performed on glass slides in the laboratory, where neither rain nor excretions from leaves can have any influence. These latter agencies may play a part, however, under field conditions, and the present paper presents the results of a study of the changes in composition undergone by films of Bordeaux mixture exposed to rain, together with accompanying changes in solubility of the copper in the film. In order to eliminate any possible influence of leaves or spores, in the present study glass plates were sprayed and exposed out-of-doors on a specially constructed rack.

The earlier work on the effect of atmospheric agencies in Bordeaux mixture has been reviewed in detail in a previous paper (5). Later investigations include those of Goldsworthy and Green (4), Reckendorfer (9), and Branas and Dulac (1). A number of these authors have stressed the connection between the gradual carbonation of the lime in Bordeaux mixture and the appearance of soluble copper. Crandall (2) analyzed the drippings from trees sprayed under orchard conditions and concluded that "copper, in small quantity, becomes soluble very soon after deposition, and continues to appear as long as any of the mixture remains upon the leaves." In a recent paper Reckendorfer (9) has studied the rôle of CO_2 and moisture in the production of soluble copper from films of Bordeaux mixture. A point which does not appear to have been previously investigated is the possible change in the copper-lime-sulphate ratio of the Bordeaux precipitate under the influence of rain. According to Martin (7) the precipitate consists essentially of hydrated copper oxide with adsorbed calcium sulphate. Prolonged washing of this precipitate in the laboratory leads finally to the production of brown or black hydrated copper oxide. In the field this change is not observed. It appeared of interest therefore to make analyses of the sprayed Bordeaux film for all three constituents, copper, calcium, and sulphate, after varying periods of exposure to rain in order to follow possible changes in the ratio of these constituents.

METHODS

Bordeaux mixture was prepared by adding a 2 per cent suspension of slaked calcium oxide or a 2.67 per cent suspension of $Ca(OH)_2$ to a 2 per

cent solution of $CuSO_45H_2O$. The resulting 4–4–50 mixture was sprayed on circular glass plates, cut from window glass and having an area of 225 sq. cm. The spray was delivered through an atomizer and regulated to a pressure of 40 mm. of mercury by means of a manometer attached to the laboratory air pressure line. The plates were sprayed as evenly as possible and the duration of spraying varied from 30 to 50 seconds per group of 7 plates, but was constant for any one experiment. This amount of spraying resulted in approximately 1.5 to 4.0 mgs. of Cu per plate.

After drying in the laboratory for about 3 hours the plates were placed out-of-doors on specially constructed weathering racks (Fig. 1). The racks
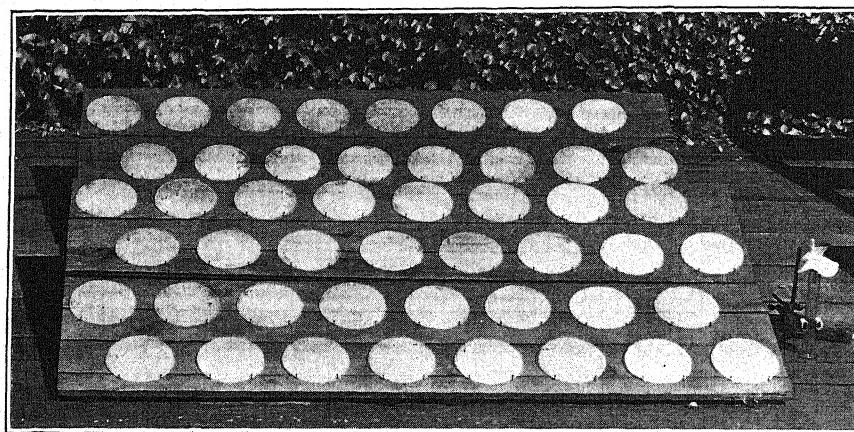
FIGURE 1. Weathering racks to hold plates sprayed with Bordeaux mixture. Rain gauge on right.

were inclined at an angle of about 20° and the upper side of the plate held about 0.5 cm. above the platform to prevent run-off water from flowing across the plate. A rain gauge was placed beside the rack and the rainfall recorded daily. Control unexposed plates were held in the laboratory. From time to time a number of plates were brought inside and determinations made of soluble and total copper and total calcium and sulphate.

Determinations in the change of the copper-calcium-sulphate ratio of the material remaining on the plates after various periods were made as follows:— Copper was determined by electrolysis, calcium gravimetrically as oxalate, and sulphate by precipitation as barium sulphate. For the initial determinations four plates were used, and the number was increased as the amount of material remaining diminished. It was not found possible to make more than three sets of determinations per experiment due to the small amount of material remaining on the plates.

In the tests involving soluble copper, each plate was placed in a moist

chamber containing 50 cc. of distilled water. The chambers were agitated overnight on the machine previously illustrated and described (6). Soluble copper was then determined by a former method (6) using the reagent sodium diethyl dithiocarbamate. In some cases the catalytic method of Quartaroli (8) was also used. These tests were usually performed in quadruplicate, employing one plate for an individual test. The insoluble copper was dissolved in dilute sulphuric acid and determined in the same manner.

RESULTS

CHANGES IN COMPOSITION

The analyses of the material remaining on the sprayed and exposed plates showed that Bordeaux mixture undergoes a marked change in com-
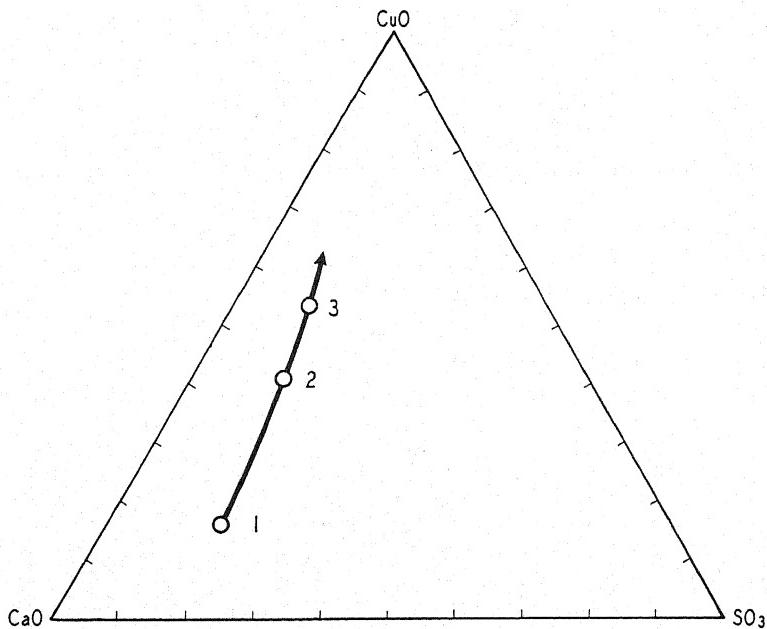
FIGURE 2. Change in composition of sprayed 4–4–50 Bordeaux mixture on weathering. Point 1 initial composition, 2 after 4 days and 0.30 inches rain, and 3 after an additional 8 days and 0.21 inches rain.

position on weathering. The leaching action of rain effects a preferential removal of lime and sulphate so that the film remaining becomes richer in copper as time goes on. The composition of such a film may be shown by means of a triangular diagram (Fig. 2) in which the relative amounts of the three components CuO, CaO, and SO_3 are plotted in mols per cent. The point 1 represents the initial composition of freshly sprayed and dried 4–4–50 Bordeaux mixture. After 4 days and 0.30 inches of rain the compo-

sition of the spray was that represented by point 2. After a further period of exposure, 8 days, and an additional rain of 0.21 inches, the composition was that indicated by point 3. It was not possible to follow the change further due to the small amount of material remaining on the plates. It is evident that sprayed Bordeaux mixture is a continually changing system, and for this reason studies made on the initial mixture may have little bearing on its properties after exposure. From the diagram it appears likely that the mixture eventually approaches the composition of pure hydrated copper oxide, unless some other factor intervenes.

CHANGES IN COPPER SOLUBILITY

Typical results of the determinations of total and soluble copper are shown in Figure 3 and Figure 4, 4–4–50 mixture. Inspection of these diagrams shows that the total copper decreases on weathering, while the soluble copper increases, reaching a maximum in about two weeks, and thereafter declines. The results in different experiments are rather variable and increases in soluble copper are noted, even in the absence of rain.

The highest amount of soluble copper observed was 0.45 mg. per plate (225 sq. cm.). This occurred after 18 days of weathering, and was 12.3 per cent of the total copper remaining. It appears that this amount of copper would give a highly toxic solution in the amount of rain water that might adhere to one plate. These results indicate that the fungicidal action of Bordeaux mixture may be dependent not only on the solubilizing action of spore excretions (6) but also on the soluble copper which arises after the spray has weathered for a period of time. It should be emphasized, however, that no weathering period is required for the spray to exert a toxic action. This gradual appearance of soluble copper must also be considered in attempting to explain the injury to fruit and foliage which occurs on many copper-sensitive plants.

Effect of change in copper-lime ratio. In order to obtain further information regarding the effect of composition on soluble copper, a comparative study of the weathering of Bordeaux mixture was undertaken, in which the copper-lime ratio of the spray varied. The experiment included 4–4–50, 4–2–50, and 4–1–50 Bordeaux mixture. Representative results are shown in Figure 4. The mixtures low in lime showed greater initial soluble copper, and on weathering increases in soluble copper appeared sooner than in the case of 4–4–50 Bordeaux. Furthermore, the low lime Bordeaux lost total copper more rapidly than the 4–4–50 mixture.

Effect of carbonation. The increases in soluble copper which were sometimes observed even when no rain had fallen require further explanation. Such changes were not observed on control plates kept in the laboratory. The other atmospheric agencies which might be expected to influence the appearance of soluble copper include carbon dioxide and dew. The follow-
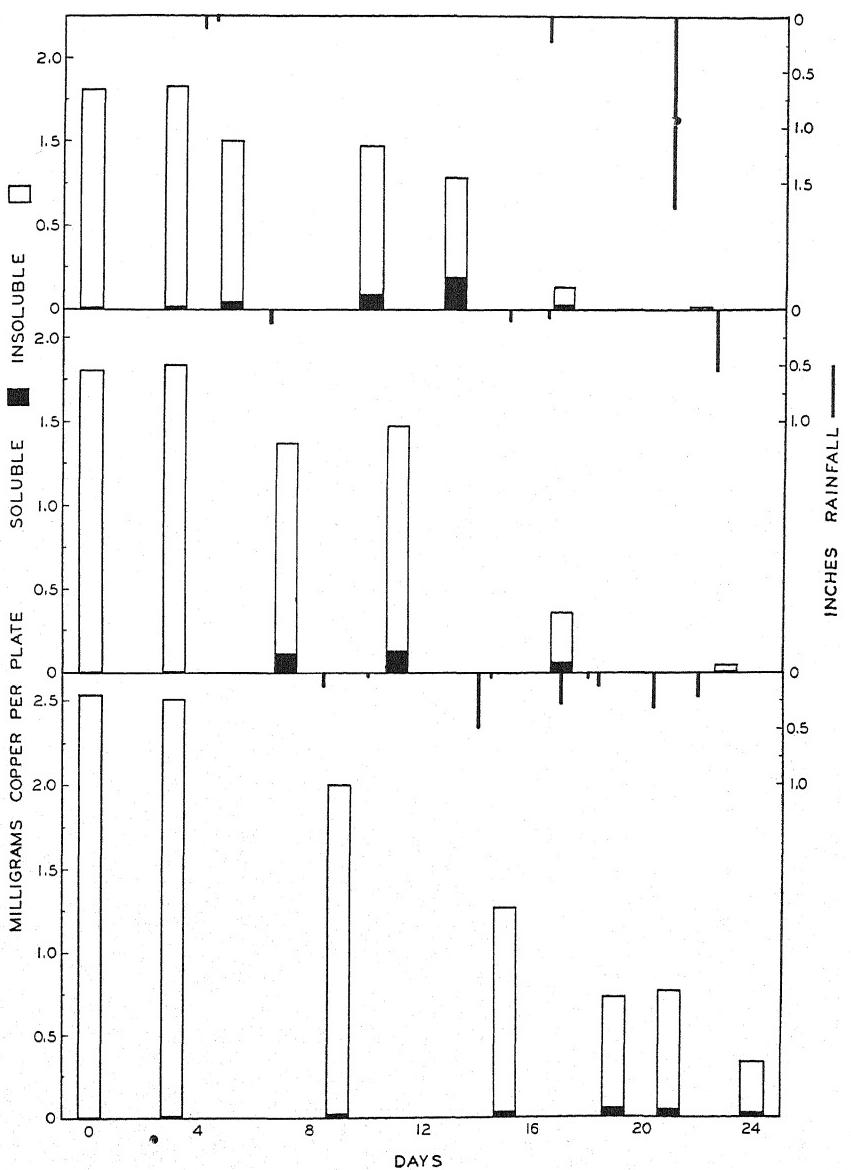
FIGURE 3. Changes in soluble and total copper of plates sprayed with 4-4-50 Bordeaux mixture after weathering. Typical experiments.
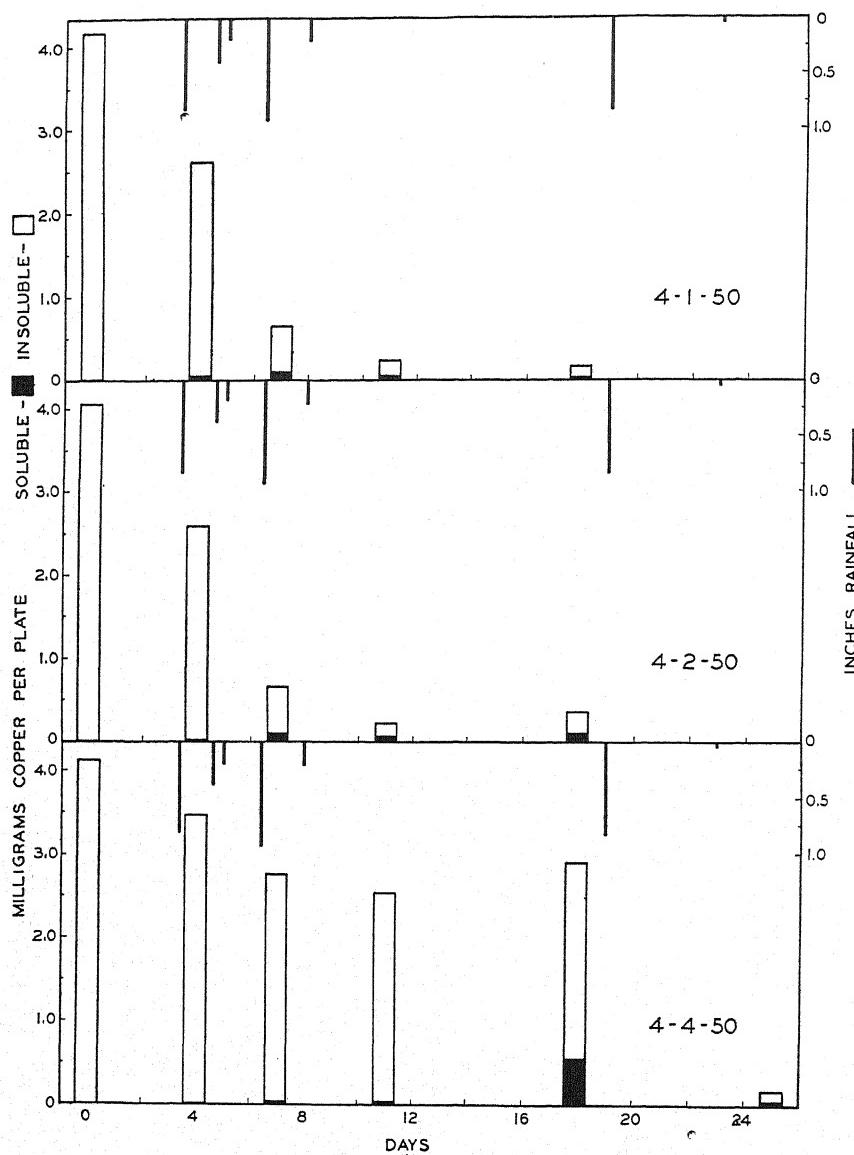
FIGURE 4. Change in soluble and total copper of plates sprayed with
varying copper-lime Bordeaux mixtures, after weathering.

ing experiment shows, however, that the carbonation of the excess lime in the Bordeaux film occurs too rapidly to account for the change observed. Sprayed plates were exposed during fair weather, and at intervals sample plates were placed on the agitator (6) with 50 cc. of distilled water and agitated for five minutes. The pH of the solution was determined with the glass electrode, and the per cent of lime carbonated calculated, assuming that the change in pH was due entirely to the neutralization of the lime by CO_2. The results are plotted in Figure 5 and show that the carbonation is 95 per cent completed in 3 hours, while the appearance of appreciable
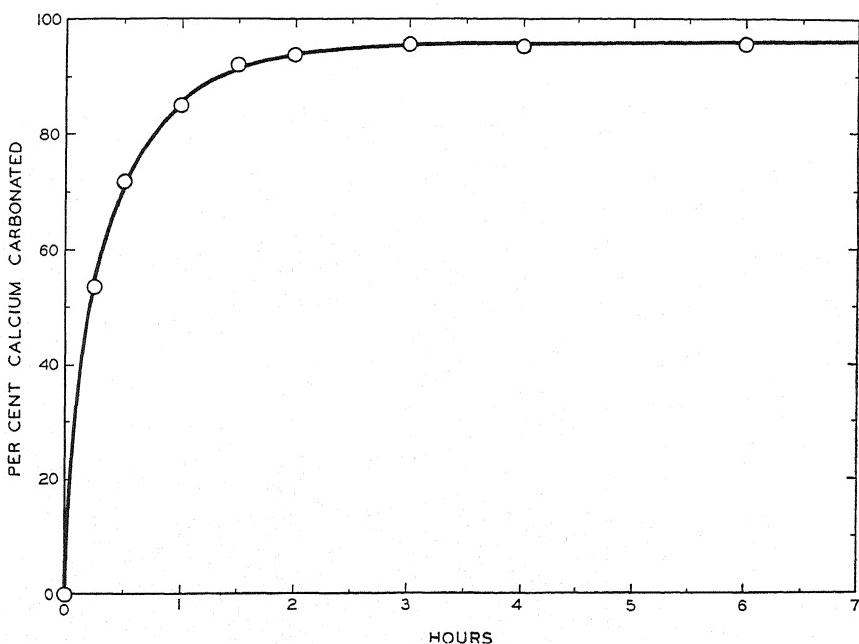
FIGURE 5. The carbonation of the calcium in sprayed Bordeaux mixture with time, as indicated by pH measurements.

amount of soluble copper requires a much longer period. Furthermore, the method of determining soluble copper involves practically complete carbonation of the free lime, since the plates are dried for several hours after spraying.

Effect of dew. Experiments were performed in which dew was collected from sprayed plates following exposure overnight. Amounts as high as 4 cc. per plate were collected. Analyses of the dew collected from the plates showed that appreciable amounts of material can be removed by this agency under certain weather conditions. In one experiment in which freshly-sprayed plates were exposed overnight, a total of 28.5 cc. of dew
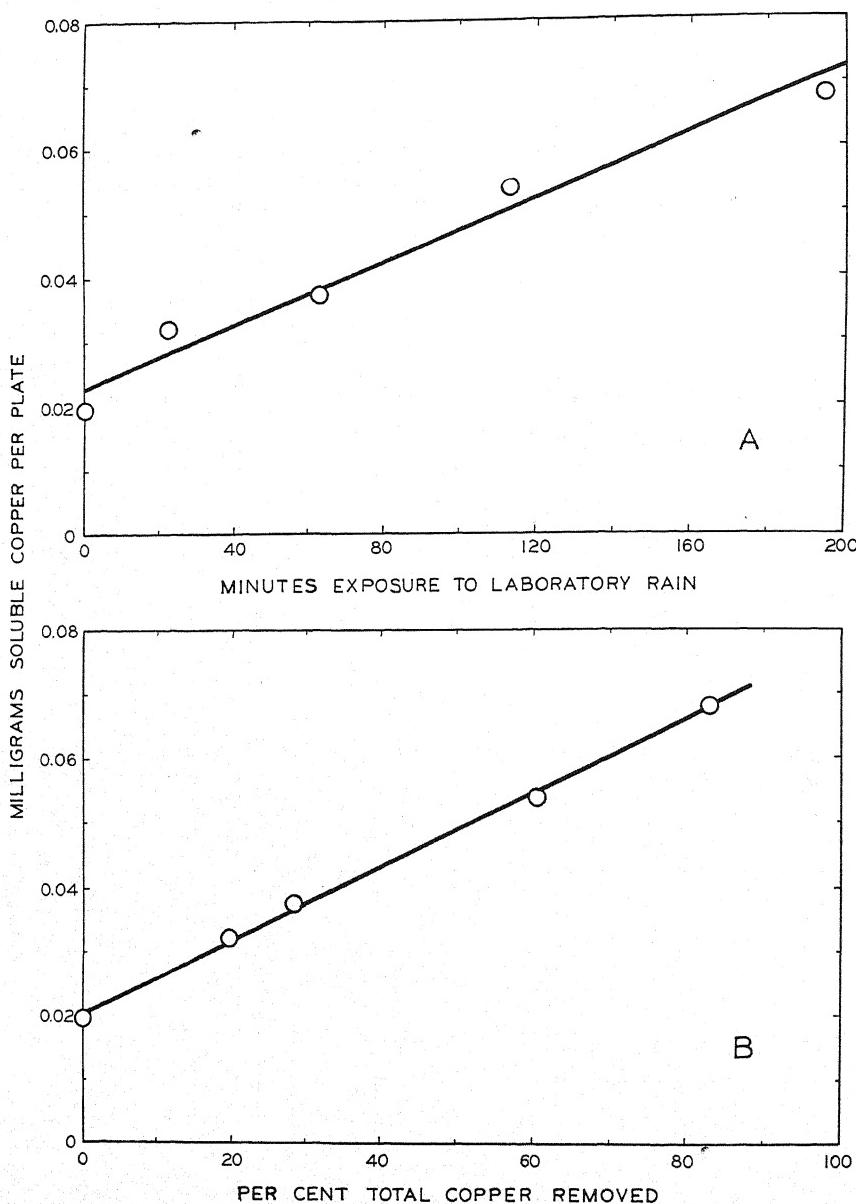
FIGURE 6. The effect of laboratory rain on sprayed Bordeaux mixture. A. Increase of soluble copper with time of exposure. B. Increase of soluble copper with loss of total copper.

was collected from 8 plates. Analyses of this dew showed that there was removed from each plate 0.0026 mg. Cu, 1.80 mg. CaO, and sulphate equivalent to 7.25 mg. of $BaSO_4$. This fact may serve to explain the increases in soluble copper which were occasionally noted, even in the absence of rain.

ARTIFICIAL WEATHERING

If the increases in soluble copper noted are due entirely to changes in composition of the precipitate as weathering proceeds, it should be possible to duplicate this effect in the laboratory by experiments with artificial rain. Accordingly 4–4–50 Bordeaux was sprayed on the circular glass plates, dried, and exposed to artificial rain. The rain was delivered through a small rose attached to the distilled water line and directed toward the sprayed plate. The pressure was adjusted to give 1 inch of rain in one minute. The plate was supported in a swinging cradle, about 25 inches below the orifice. It was found that an inch of rain under these conditions was far less efficient in removing the spray from the plates than a corresponding amount out-of-doors. The rain out-of-doors strikes with greater impact and the distribution of the drops is different. After varying periods of exposure, the plates were dried and the soluble and total copper determined. The results are shown in Figure 6. It is evident that the leaching effect of the artificial rain brings about a marked increase in the soluble copper. The maximum amount of soluble copper obtained approximates that obtained in some of the outdoor experiments.

Experiments in which the Bordeaux precipitate was washed either by centrifuging, or by washing on a Buchner funnel, until incipient darkening of the precipitate occurred, did not lead to such large increases in soluble copper. Apparently the leaching effect of the artificial rain is much greater than ordinary washing. Carbonation of the precipitate with a current of CO_2, either before or after washing, did not lead to such increases in soluble copper as were observed in the experiments with artificial rain.

DISCUSSION

The investigations described in this paper lead to the conclusion that sprayed films of Bordeaux mixture undergo a continual change in composition under the influence of rain and dew. The excess lime in the mixture is carbonated quite rapidly, and the subsequent leaching effect of rain removes calcium and sulphate at a greater rate than copper, leaving a residue relatively richer in copper as weathering proceeds. This change in composition of the precipitate is accompanied by the appearance of increased amounts of soluble copper, which may furnish toxic solutions under some conditions. These results may be duplicated in the laboratory, but only if sprayed and dried films of Bordeaux mixture are used for the experiment. Prolonged washing of the unsprayed Bordeaux precipitate by centrifuging

or on a Buchner funnel leads only to darkening of the precipitate, without such increases of soluble copper as are observed with sprayed films. In addition, the films do not darken on washing as does the unsprayed precipitate, and these facts strongly indicate that the properties of the sprayed films are chemically and physically different from those of the unsprayed precipitate.

The rapid carbonation of the excess lime in the film cannot explain the appearance of soluble copper, since the latter does not appear until long after the initial carbonation is complete. The question remains as to whether a slower prolonged action of CO_2 on the copper compounds in the film is responsible for the increases in soluble copper observed. This view has been emphasized by Reckendorfer (9), who gives a series of equations for the action of CO_2 on the precipitate leading finally to $CuSO_4$, $Cu(HCO_3)_2$, and $Ca(HCO_3)_2$. However the equilibrium relations between hydrated copper oxide and CO_2 are such that even the normal carbonate $CuCO_3$ is never obtained by ordinary procedures, and it seems unlikely that the acid carbonate $Cu(HCO_3)_2$ could be formed under as low pressures of CO_2 as exist in the atmosphere. Free (3) has studied the solubility of basic copper carbonate under varying pressures of CO_2 and found 8.3 p.p.m. of copper in water containing 157 p.p.m. of CO_2, while in water to which no extra CO_2 was added the solubility was 1.5 p.p.m., at 30° C.

Reckendorfer also considers that the reaction forming $Cu(HCO_3)_2$ is reversible so that during dry periods the solubility of the copper is lowered, while during wet periods it is increased. The present authors found no evidence of such an alternating solubility. In our experiments the solubility gradually increased on weathering until the total copper remaining on the plates was only about 12 per cent of the original value.

Martin (7) has presented evidence showing that the Bordeaux precipitate is essentially hydrated cupric oxide stabilized by adsorbed calcium oxide and sulphate. If this is the case, or if the mixture constitutes a solid solution, which is indistinguishable from an adsorption complex by physicochemical means, then it is reasonable to expect that the solubility of the copper would increase as the lime and sulphate were removed by washing. The definite compounds which have been assumed to be present in the precipitate by some investigators, have never been shown to exist with certainty, and therefore serve no useful purpose in attempting to explain the properties of the mixture.

SUMMARY

1. When glass plates are sprayed with 4–4–50 Bordeaux mixture, and exposed outdoors, the sprayed film undergoes a change in composition under the leaching influence of rain and dew, leading to a mixture relatively richer in copper.

2. This change in composition is accompanied by an increase in soluble copper. The highest amount observed was 0.45 mg. per plate (225 sq. cm.), when the plate was agitated with 50 cc. of water. Carbonation of the excess lime was complete in a few hours, as judged by pH measurements, but the increases in soluble copper did not occur until much later.

3. The results could be duplicated in the laboratory using artificial rain, but only if a sprayed, dried film of Bordeaux were used. Washing the Bordeaux precipitate in bulk by centrifuging, or on a Buchner funnel, did not lead to substantial increases in soluble copper.

4. When Bordeaux mixtures low in lime were submitted to the leaching action of rain, soluble copper appeared sooner than with a 4–4–50 mixture.

5. Treatment of the sprayed films with CO_2, either wet or dry, did not lead to much increase in soluble copper. It is considered that the increases in soluble copper observed can be best explained by assuming that the weathered Bordeaux precipitate is an adsorption complex, or a solid solution containing copper, lime, and sulphate, the copper of which is soluble in water to an extent which varies with its composition.

6. The appearance of small amounts of soluble copper must be considered as a factor in connection with foliage injury, as well as in fungicidal action where it may supplement the solvent action of spore excretions previously reported.

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APPARATUS FOR STUDYING EFFECTS OF LOW CONCENTRATIONS OF GASES ON PLANTS AND ANIMALS

CARL SETTERSTROM AND P. W. ZIMMERMAN[1]

In the study of the effects of long exposure of plants or animals to low concentrations of a particular gas, precise control of the gas concentration with a minimum of attention, and at least partial control of other important factors such as temperature and humidity, are essential requirements.

Apparatus to meet such requirements has evolved largely from studies of the effects of sulphur dioxide on plants. In 1915 the Selby Smelter Commission (3) described a method of fumigating plants which was a great advance over methods of previous investigators who had merely added a measured amount of sulphur dioxide to a known volume of air inside a glass case which contained the plants. Wells of the Selby Commission showed that the sulphur dioxide was rapidly absorbed by the glass walls, by the plant, and by the soil with consequent decrease in concentration. He, therefore, devised a method of blowing air to which had been added an approximately known amount of SO_2, into long, low cabinets which were placed over the plants to be fumigated.

This type of cabinet, however, had several disadvantages. It was not possible to maintain the same concentrations at inlet and at the far end of the cabinet and it was difficult to control temperature and humidity.

O'Gara (4) improved the Selby Commission apparatus considerably. He metered the SO_2 through empirically calibrated flowmeters and measured the volume of air going into the cabinets with a pitot tube. His cabinets were six feet square with a height varying upwards from four feet depending on the material being treated. The air-gas mixture was blown in at the top of the cabinet and distributed uniformly by means of radial baffles. As no exit was provided, air leaked out around the bottom of the cabinet. SO_2 concentrations in the cabinets were checked intermittently by means of the iodine titration method developed by Selby Smelter Commission (3, p. 189).

Hill and Thomas (2) used apparatus similar to that of O'Gara with several important exceptions. The cabinets were made more nearly gas-tight and an exit was provided for the gas mixture. The volume of air entering and leaving the cabinets was measured by means of anemometers

inserted in the inlet and outlet pipes. Apparatus for metering the SO_2 was inclosed and thermostated. Humidity control was effected by blowing an atomized spray of water, augmented with steam when necessary, into the intake pipe.

The most important contribution to this problem in recent years has been the development by Thomas and his associates (7, 9, 10) of automatic apparatus, autometers, for continuously analyzing air containing low concentrations of sulphur dioxide. This apparatus automatically draws a given volume of the air-SO_2 mixture through a known volume of absorbing solution in a given length of time. The absorbing solution, a slightly acidulated solution of hydrogen peroxide, oxidizes the SO_2 to sulphuric acid, and the change in conductivity of the absorbent is recorded on a Leeds and Northrup Micromax electrical conductivity recorder.

By changing the absorbing solution, and making minor alterations in the apparatus, the Thomas autometers can be adapted to record automatically concentrations of many other gases. Thomas describes a similar apparatus for determining CO_2 (8, 11). Walter (12) reports that the following gases have been determined by measuring conductivity of absorbing solution: H_2S [absorbed in $Pb(OAc)_2$], SO_2 (absorbed in $K_2Cr_2O_7$), NH_3 (absorbed in pure H_2O or in very dilute H_2SO_4), CO_2 (absorbed in NaOH), O_2 (absorbed in potassium pyrogallate), C_2H_2 (absorbed in ammoniacal Cu_2Cl_2), CO (absorbed in Cu_2Cl_2), N oxides (absorbed in $FeSO_4$), Cl and Br (absorbed in H_2O or in alkaline H_2O_2), and SCl_2 (absorbed in KOH).

Further improvements in design of fumigating cabinets have been described by Zimmerman and Crocker (13). Swain and Johnson (6) washed and humidified the air before passing it into their fumigating system.

In designing the apparatus described in this paper the writers were particularly anxious to obtain precise control, with a minimum of care, of gas concentrations approximating 0.10 part of the gas per million parts of air by volume (p.p.m.) for fumigations lasting several weeks.

The apparatus (Fig. 1) consists of duplicate sets, one for fumigating the test material, the other for controls. Each set includes a cabinet in which the material is placed during the experiment; a variable speed exhauster blower and an orifice meter for precise control of the volume of air passing through the cabinet; specially designed scrubbers attached to the intake side of the blowers to rid the air of SO_2 and other impurities, before allowing it to enter the system, and to effect a partial control of the temperature and the humidity in the cabinet; a Thomas autometer and a conductivity recorder for determining sulphur dioxide content of the air in the cabinet; a humidigraph for recording the temperature and humidity in the cabinet.

Accurate metering of the gas entering the test cabinet is made possible by the use of calibrated capillary flowmeters, a reducing regulator, a sen-
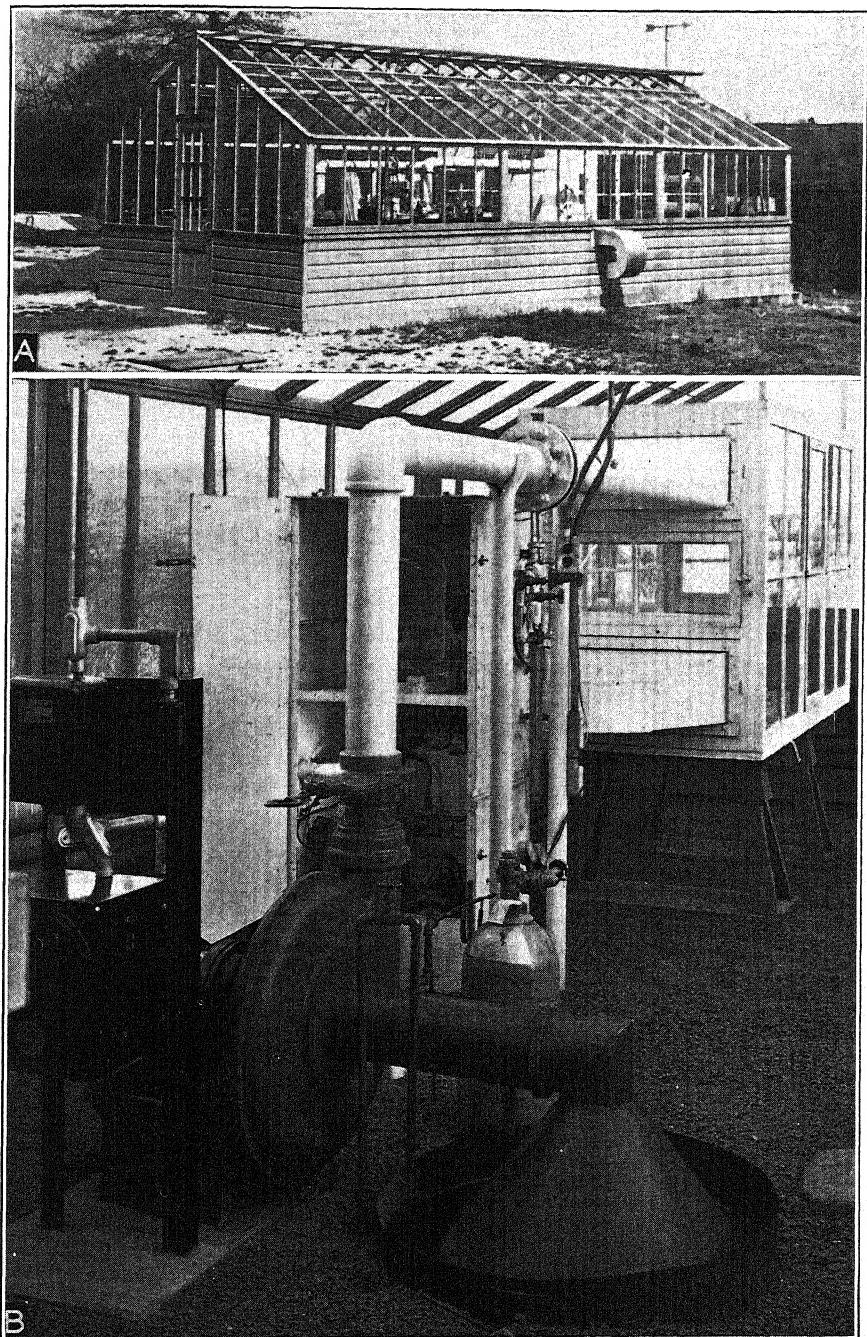
FIGURE 1. Part of the apparatus used for studying the effects of gases on plants and animals. A, Greenhouse which houses most of the apparatus. B, Close-up of scrubber and metering devices with cabinet and autometer in the background.

sitive needle valve, and cylinders containing special mixtures of air and the gas.

All the apparatus, except the conductivity recorders, is housed in a small greenhouse which is provided with automatic temperature control. The conductivity recorders are installed in a laboratory within the Institute building.

DETAILED DESCRIPTION OF APPARATUS

Gas-air mixture. In working with concentrations approximating 0.10 p.p.m., unless the volume of air used is greater than 350 cubic feet per minute (c.f.m.), the volume of gas metered into the system is less than a cc. per minute. Accurate control of the gas flow is facilitated by using a mixture of 10 or 5 per cent of the gas and 90 or 95 per cent air, thereby increasing the volume of gas metered 10 or 20 times with consequent greater precision. Commercial mixtures of numerous gases with air are available on the market. The gas mixtures used at the Institute were prepared by the Ohio Chemical and Manufacturing Company, Cleveland, Ohio.

When using concentrations greater than 1.00 p.p.m. it is possible to obtain precise control with the undiluted gas.

Metering of the gas. Delicate control of the gas flow is made possible by means of a diaphragm reducing regulator and a sensitive needle valve. The reducing regulator, equipped with high pressure gauge which measures the pressure within the cylinder, and low pressure gauge which registers the pressure on the upstream side of the needle valve, enables the maintenance of a constant pressure on the needle valve despite fluctuations in tank pressure.

Calibration of capillary flowmeters. The gas is measured by passing it through a calibrated capillary flowmeter. For use with the gas-air mixture, a series of flowmeters are calibrated with air. Since flow is a function of the square root of density, the figures obtained for air are multiplied by the proper factor to correct for the presence of the gas. Air from Institute pressure line, dried by passing over calcium chloride, was passed through the flowmeter at a given uniform rate, as determined by manometer reading, and collected over water at atmospheric pressure in calibrated leveling bottles. The time for a given volume of air to pass through the flowmeter at a given manometer reading was determined with a stop watch. Volume readings were corrected for water vapor pressure.

Since velocity through an orifice varies with the square root of the manometer reading, the calibration curve of velocity against manometer reading becomes a straight line when plotted on logarithmic coordinates.

The calibrations were checked by using a mixture of 5 per cent SO_2 and 95 per cent air, absorbing the SO_2 in a weak solution of hydrogen per-

oxide and titrating with standard alkali using methyl red as an indicator.

Blowers. The air is blown into the fumigating cage by means of a clockwise vertical discharge blower with 18-1/4″ diameter wheel, handling up to 600 cubic feet per minute. The blower is driven by a 1-1/2 H.P., 1750 r.p.m., 3 phase, 60 cycle, 208 volt, ball bearing motor with an automatic push button starter and speed regulator.

The speed regulator and a Rockwell sealed gas blast gate which is located on the discharge side of the blower together make possible precise regulation of the air flow.

Orifice meter. The volume of air entering the fumigating cabinet is measured by means of an indicating orifice meter. The orifice is set in the 4-inch cast iron pipe, which delivers the air from blower to fumigating cabinet, between two flanges which contain the upstream and downstream taps for determining differential pressure. There are 38 inches of straight pipe in front of the orifice to straighten out any irregularities in flow. Seventeen inches of straight pipe are behind the orifice plate to insure complete restoration of pressure before the air stream is diverted from its path.

Two sizes of orifices are used: one for measuring 20 to 100 c.f.m., the other for measuring 100 to 500 c.f.m. The orifice plates can easily be interchanged.

Orifice coefficients, supplied by the manufacturer, enable plotting of the calibration curve which assumes the form of a straight line on logarithmic coordinates. The effects of temperature and humidity on the specific gravity of the air being measured are not of sufficient magnitude to require correction.

The orifice meter has several advantages over other methods of measuring flow of gases. It has no moving parts to get out of order or require repeated calibration, cost is relatively low, and different ranges of flow can be measured merely by changing the orifice plates.

For a discussion of the theory of the orifice meter and for a comparison with other methods of measuring flow, the reader is referred to a paper by Dodge (1).

Fumigating cabinets. The fumigating cabinets are 46 inches high × 34 inches wide × 82 inches long with cubic capacities of 74.2 cubic feet. They are constructed of single strength, B quality, flat drawn glass. The bottoms of the cabinets are galvanized iron covered with two coats of white enamel. Each cabinet has a removable side door and removable end sections, three sections constituting an end. The cabinets are very convenient for treating animals or potted plants. Measurements in sunlight with a Lange photocell with neutral filters show that light intensity is reduced about 16 per cent on passing through the greenhouse glass and about 15 per cent on passing through the cabinet glass. The total reduction in light intensity

on passing through both the greenhouse and the cabinet glass is, there-fore, about 29 per cent.

The air passes from the 4-inch pipe through a wrought iron truncaded hollow pyramid 23 inches long into a $10'' \times 27''$ opening at the top of one end of the cage. The air leaves the cabinet at a similar opening at the bottom of the same end and is shunted through a $10'' \times 27''$ wrought iron vent to the outside of the greenhouse where a $180°$ elbow turned back on the house reduces possible back draft.

Careful dispersion tests indicate that the air blown into the cabinet is uniformly dispersed throughout.

Thomas autometers. The air-gas mixtures are sampled automatically by means of the Thomas autometers (7, 9, 10). One autometer usually is connected with the inlet and with the outlet of the test cabinet, another with the inlet of the control cabinet and with the atmosphere outside the greenhouse.

Changes in conductivity of the absorbing solution are measured by means of platinum electrodes and recorded on the chart of a Leeds and Northrup Micromax conductivity recorder. The recorder is equipped with a control mechanism which is used to ring a bell when the conductivity reaches a certain point, thus giving warning when the gas concentration in the cabinet becomes too high.

The electrode-recorder system including the temperature compensator must be calibrated for the gas that is to be measured. For use with sulphur dioxide, the system was calibrated with H_2SO_4 of normalities ranging from 0.001 to 0.00001. Knowing the normality corresponding to a given resistance on the recorder scale, and knowing the volume of absorbing solution, it is a simple matter to determine the equivalent volume of SO_2. With this figure, and that of the volume of air used to supply the equivalent volume of SO_2, the figure for p.p.m. is readily obtainable. For example, assume 6.81 cubic feet of a mixture of SO_2 and air at $20°$ C. and 755 mm. of mercury are drawn through 100 cc. of absorbing solution with original resistance of 4000 ohms, and that the resistance of the solution then decreases to 2600 ohms. Consulting the calibration curves, resistances of 4000 ohms and of 2600 ohms are found to correspond, let us assume, to 0.0000582 N and 0.0000870 N sulphuric acid. Neglecting the correction for barometric pressure, the equivalent volume of SO_2 is then found to be

$$(0.0000870 - 0.0000582) \times \frac{100}{1000} \times \frac{22,400}{2} \times \frac{293}{273} = 0.0346 \text{ cc.}$$

A volume of 0.0346 cc. of SO_2 per 6.81 cubic feet of air gives a concentration of 0.18 p.p.m. If the temperature and the volume of air sampled per period are maintained constant, parts per million become directly proportional to normality and can be read from the recorder chart by means of calibrated rules.

Theoretical concentrations, established with the capillary flowmeters and the orifice meter, agree very well in all cases with concentrations as recorded by the autometer-recorder system.

Scrubbers. In the course of experimental work with low concentrations of sulphur dioxide, it was soon discovered that considerable concentrations
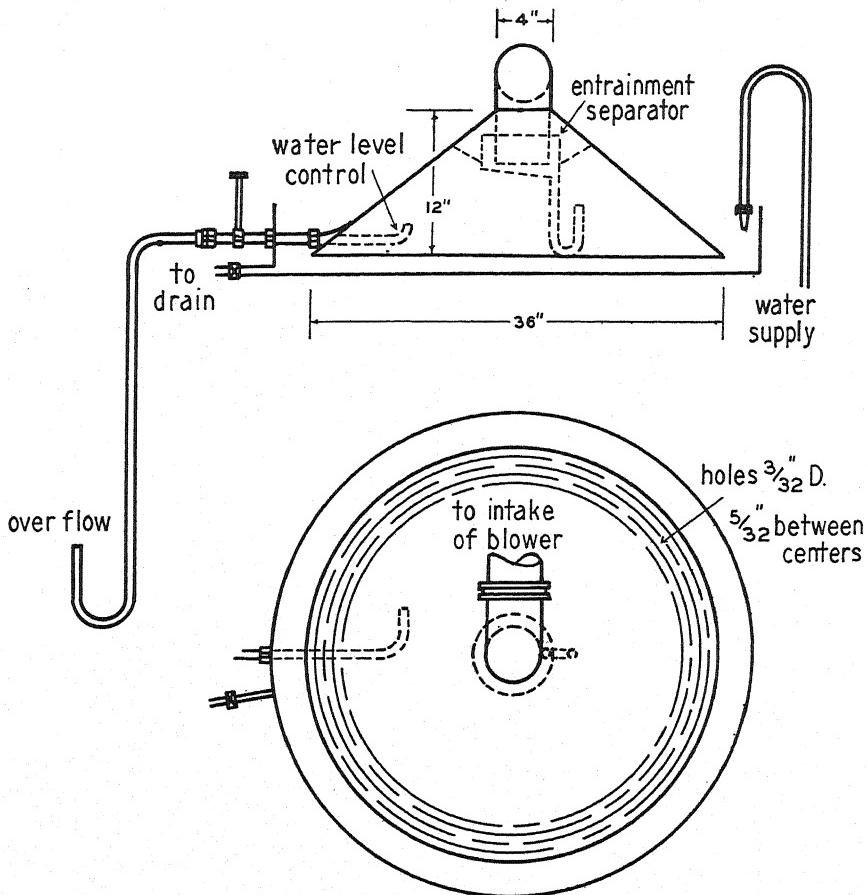
FIGURE 2. Diagram of scrubber used to remove sulphur dioxide from the air which is passed into the fumigating systems.

of that gas prevail in the atmosphere at Boyce Thompson Institute (5).

In order to rid the air of sulphur dioxide before allowing it to pass into the fumigating systems, specially designed scrubbers were attached to the intake side of the blowers. The SO_2 is removed and partial humidity and temperature control is effected by bubbling the air through a constantly changing supply of tap water (pH 7.1 to 7.3). Details of this SO_2 absorption system are shown in Figure 2. The SO_2 removal efficiency is from 93
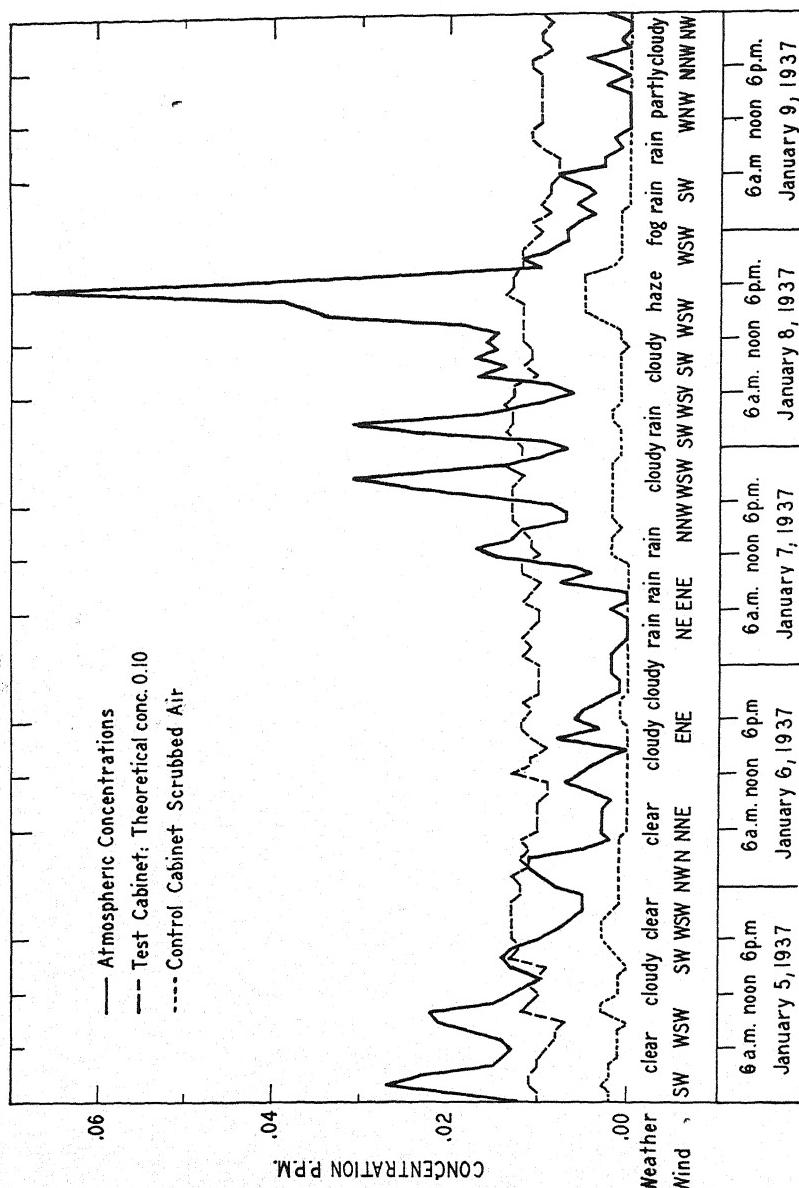
FIGURE 3. Graph showing variations in sulphur dioxide content of atmosphere, of fumigating cabinet, and of control cabinet.

to 100 per cent (Fig. 3). Other water soluble gases, dust particles, etc. are also removed by the scrubbers.

APPLICATIONS OF APPARATUS

The apparatus described has been used for studies of the effects of sulphur dioxide on plants and on guinea pigs and mice. It has given satisfactory service for over a year of almost continuous operation with concentrations of SO_2 varying from 0.10 to 60 p.p.m. Experiments have run for 25 days at 0.10 p.p.m. without fluctuating in concentration more than ±0.02 p.p.m.

With minor changes the apparatus can be adapted to study the effects of many other gases such as carbon dioxide, ethylene, chlorine, hydrochloric acid gas, and ammonia.

SUMMARY

An apparatus for studying the effects of long exposure of plants or animals to various gases is described in detail. The apparatus has given satisfactory service for over a year of almost continuous operation with concentrations of SO_2 varying from 0.10 to 60 p.p.m.

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SULPHUR DIOXIDE CONTENT OF AIR
AT BOYCE THOMPSON INSTITUTE

CARL SETTERSTROM AND P. W. ZIMMERMAN

In the course of experimental fumigations with low concentrations of sulphur dioxide, it was soon discovered that measurable concentrations of that gas prevail in the atmosphere at Boyce Thompson Institute. In view of the importance of sulphur dioxide as an atmospheric contaminant, and in view of the wide disagreement as to the harmful effects to plants of low concentrations of the gas, it was felt that a continuous record of sulphur dioxide concentrations in the atmosphere at the Institute would be of considerable interest. Such a record has been kept from November 1, 1936 to November 1, 1937, with minor interruptions, and is summarized herein.

The presence of considerable quantities of sulphur dioxide in the atmosphere in and about industrial cities has been established by Betz, Holden, and Handy (1), Meller (4), Holmes, Franklin, and Gould (3), the Air Hygiene Foundation (5), and others. These investigators, however, made individual determinations of SO_2 at varying intervals, none of them attempting to keep a continuous record of the prevailing concentrations.

METHODS

Automatic, continuous determination of sulphur dioxide concentrations was made possible by use of the sampling mechanism, or autometers, developed by Thomas and his associates (8, 9, 10). Concentrations were continuously recorded on Leeds and Northrup Micromax conductivity recorders. The autometer-recorder system is described in a previous paper (7). The system was calibrated with solutions of sulphuric acid of known normality, and the calibrations checked by sampling known concentrations of SO_2.

This system is not specific for sulphur dioxide because other acid gases will bring about a similar change in conductivity of the absorbing solution. The iodine titration method developed by Griffin and Skinner (2) was, therefore, used at intervals for independent determinations. The consistent agreement between the iodine and conductivity methods supports the assumption that the gas measured by the conductivity system was sulphur dioxide.

The autometers were operated on a 32-minute sampling schedule. Chart readings were made, therefore, every 32 minutes except where the slope of the conductivity recording indicated sharp variations in concentration within that period. In the latter event, average concentrations were determined over shorter periods of time.

SUMMARY OF DETERMINATIONS

The results of the continuous sulphur dioxide determinations are summarized in Table I. For the year period, the average concentration, including zero readings, was 0.033 part of sulphur dioxide per million parts of air by volume (p.p.m.). Maximum concentration recorded was 0.75 p.p.m. Sulphur dioxide was present, in concentrations of 0.01 p.p.m. and over, 62.2 per cent of the time.

TABLE I

SUMMARY OF CONCENTRATIONS OF SO_2 IN THE ATMOSPHERE, AT BOYCE THOMPSON INSTITUTE, YONKERS, NEW YORK, NOVEMBER 1, 1936 TO NOVEMBER 1, 1937

Month	Hours recorder was operating	% time conc. was 0.01 p.p.m. and above	% time conc. was 0.10 p.p.m. and above	% time conc. was 0.20 p.p.m. and above	% time conc. was 0.30 p.p.m. and above	% time conc. was 0.40 p.p.m. and above	Max. conc.	Av. conc.
November	707	89.6	7.3	0.5	0.1	0.1	0.44	0.040
December	726	79.5	15.6	2.5	0.1	0.1	0.54	0.049
January	735	82.3	14.9	3.4	1.4	0.4	0.69	0.049
February	634	75.3	12.2	3.1	1.1	0.0	0.75	0.046
March	742	67.0	6.5	1.0	0.1	0.1	0.41	0.030
April	720	53.8	7.4	0.7	0.0	0.0	0.25	0.029
May	739	57.6	5.7	0.5	0.1	0.0	0.33	0.029
June	697	42.3	1.9	1.5	0.0	0.0	0.20	0.016
July	741	38.1	5.8	0.6	0.1	0.0	0.34	0.020
August	478	51.1	9.0	0.4	0.0	0.0	0.21	0.028
September	720	50.1	7.4	1.9	0.3	0.0	0.30	0.029
October	360	53.3	6.8	0.3	0.0	0.0	0.29	0.026
November 1, 1936 to November 1, 1937	7999	62.2	8.4	1.4	0.28	0.06	0.75	0.033

Seasonal variation of concentration shows that heating plants are a source of considerable of the sulphur dioxide in the air around the Boyce Thompson Institute. So-called "more important occurrences" of the gas are listed at considerable length in Table II in order to give a fairly complete picture of the magnitude and duration of the visitations of the gas.

In connection with the effects of sulphur dioxide on plants grown in greenhouses, it is important to know the relationship of greenhouse concentrations to atmospheric concentrations. A study of this relationship was made and the results are summarized in Table III.

The greenhouse used for this study is a semi-iron greenhouse 14'4" wide by 25'5-3/4" long, with two complete glass gables. The study of greenhouse concentrations was made during January, and the ventilators were completely closed during the night and partly opened from about 9:00 A.M. to 3:30 P.M. in accordance with the accepted practice in greenhouse management.

TABLE II

LIST OF THE MORE IMPORTANT OCCURRENCES OF SO₂ IN THE ATMOSPHERE AT
BOYCE THOMPSON INSTITUTE
NOVEMBER 1, 1936 TO NOVEMBER 1, 1937

Date	Time	Total hrs. of gas	Av. conc. in p.p.m.	Max. conc. in p.p.m.
Nov. 1– 6	12:01 A.M.– 8:54 A.M.	128.9	0.055	0.18
Nov. 7– 9	1:04 P.M.– 1:42 P.M.	48.6	0.041	0.36
Nov. 9–10	4:23 P.M.–12:16 P.M.	19.9	0.064	0.17
Nov. 11–12	10:50 A.M.– 4:38 A.M.	17.8	0.097	0.13
Nov. 12–14	7:17 A.M.–12:23 A.M.	41.1	0.084	0.36
Nov. 14–15	3:04 A.M.– 3:47 A.M.	24.7	0.076	0.20
Nov. 19–21	2:16 P.M.–10:12 A.M.	43.9	0.059	0.23
Nov. 24–26	2:13 A.M.– 4:55 A.M.	50.8	0.041	0.44
Nov. 28–29	12:36 A.M.– 3:19 A.M.	26.7	0.047	0.32
Nov. 30–Dec. 1	11:18 P.M.–10:35 A.M.	11.3	0.027	0.31
Dec. 1– 2	5:34 P.M.– 6:27 A.M.	12.9	0.023	0.46
Dec. 2– 3	9:21 P.M.– 4:09 P.M.	18.8	0.056	0.15
Dec. 3– 4	5:46 P.M.– 9:21 P.M.	15.6	0.053	0.14
Dec. 5– 6	8:28 A.M.– 2:35 A.M.	18.1	0.037	0.28
Dec. 6– 7	5:49 A.M.– 4:22 A.M.	22.5	0.061	0.11
Dec. 7– 8	9:02 P.M.– 9:56 A.M.	12.9	0.041	0.21
Dec. 8– 9	4:23 P.M.– 2:04 A.M.	9.7	0.045	0.33
Dec. 10	12:32 P.M.– 9:46 P.M.	9.1	0.106	0.53
Dec. 10–12	10:18 P.M.– 8:58 A.M.	34.7	0.060	0.55
Dec. 12	9:30 A.M.–11:36 P.M.	13.9	0.085	0.17
Dec. 13–14	12:08 A.M.– 4:40 P.M.	40.5	0.081	0.32
Dec. 14–15	6:18 P.M.– 5:02 P.M.	22.7	0.076	0.25
Dec. 17	4:45 A.M.–10:44 P.M.	18.1	0.073	0.35
Dec. 17–19	11:16 P.M.– 1:02 A.M.	25.8	0.049	0.11
Dec. 21–23	4:47 P.M.– 6:13 A.M.	37.4	0.044	0.11
Dec. 23–24	8:58 A.M.– 6:12 P.M.	33.3	0.114	0.25
Dec. 25–26	11:16 A.M.–10:28 P.M.	35.2	0.109	0.51
Dec. 27–28	5:28 A.M.– 3:03 A.M.	21.6	0.085	0.19
Dec. 31	6:32 A.M.– 5:12 P.M.	10.7	0.141	0.27
Jan. 3, 1937	8:08 A.M.–12:14 P.M.	4.1	0.096	0.18
Jan. 4– 6	4:32 P.M.– 8:16 A.M.	39.7	0.104	0.30
Jan. 7– 9	8:22 A.M.–10:50 A.M.	50.5	0.138	0.69
Jan. 11	8:02 A.M.– 1:12 P.M.	5.2	0.064	0.13
Jan. 11–12	6:02 P.M.–11:42 A.M.	17.8	0.095	0.20
Jan. 13–15	1:52 P.M.– 6:34 A.M.	40.7	0.079	0.26
Jan. 15	7:07 A.M.– 9:37 P.M.	14.4	0.052	0.20
Jan. 18–19	10:25 A.M.–11:18 A.M.	25.1	0.042	0.16
Jan. 21–24	7:29 A.M.– 3:14 P.M.	79.9	0.089	0.33
Jan. 24–25	5:54 P.M.– 1:40 P.M.	19.7	0.051	0.20
Jan. 25–27	2:12 P.M.– 5:57 A.M.	39.8	0.037	0.11
Jan. 27–28	9:09 A.M.– 8:37 A.M.	23.5	0.046	0.12
Jan. 29	2:27 P.M.– 7:15 P.M.	4.8	0.083	0.13
Jan. 29–30	7:47 P.M.–12:35 A.M.	4.8	0.074	0.15
Jan. 30	3:15 A.M.–10:47 A.M.	7.5	0.060	0.10
Jan. 30–31	4:39 P.M.– 2:15 A.M.	9.6	0.050	0.11
Feb. 2– 5	3:41 P.M.– 2:10 A.M.	58.5	0.057	0.14
Feb. 5– 6	4:22 P.M.–10:30 A.M.	18.1	0.043	0.10
Feb. 6– 7	12:38 P.M.– 7:50 A.M.	19.2	0.061	0.21
Feb. 9–10	3:51 A.M.–12:34 P.M.	32.5	0.109	0.36
Feb. 11–14	1:06 P.M.– 3:46 A.M.	62.7	0.076	0.33
Feb. 14	9:38 A.M.– 3:30 P.M.	5.9	0.060	0.19
Feb. 16–17	8:15 P.M.– 6:13 P.M.	21.9	0.066	0.11
Feb. 19–20	1:47 A.M.–11:15 P.M.	45.3	0.106	0.75
Feb. 23–24	4:08 P.M.– 5:28 A.M.	13.3	0.090	0.24
Feb. 24–25	9:02 P.M.–11:32 A.M.	14.4	0.131	0.38

TABLE II (*Continued*)

Date	Time	Total hrs. of gas	Av. conc. in p.p.m.	Max. conc. in p.p.m.
Mar. 3– 4	10:19 P.M.– 8:02 P.M.	21.7	0.111	0.25
Mar. 6	5:57 P.M.–11:17 P.M.	5.3	0.059	0.10
Mar. 8	12:21 A.M.–12:37 P.M.	12.3	0.060	0.13
Mar. 8– 9	2:59 P.M.– 1:39 A.M.	10.7	0.073	0.17
Mar. 11–12	4:38 P.M.– 7:02 A.M.	14.4	0.047	0.15
Mar. 12	9:58 A.M.– 3:50 P.M.	5.9	0.066	0.12
Mar. 12–13	4:22 P.M.– 1:10 P.M.	20.8	0.128	0.41
Mar. 15–16	10:12 P.M.– 6:44 A.M.	8.5	0.071	0.11
Mar. 20	5:11 A.M.–12:42 P.M.	7.5	0.088	0.18
Mar. 24	4:45 A.M.– 2:59 P.M.	10.1	0.077	0.27
Mar. 25–26	4:16 P.M.– 7:44 A.M.	15.5	0.051	0.10
Mar. 31	7:13 A.M.– 3:47 P.M.	8.4	0.050	0.13
Mar. 31–Apr. 1	4:19 P.M.– 7:15 A.M.	14.9	0.093	0.26
Apr. 1– 2	7:07 P.M.– 9:43 P.M.	26.7	0.049	0.15
Apr. 3	3:03 A.M.– 4:57 P.M.	13.9	0.044	0.11
Apr. 6	4:17 A.M.– 3:20 P.M.	11.2	0.106	0.22
Apr. 8	4:48 A.M.– 8:20 P.M.	15.5	0.040	0.15
Apr. 9–10	10:12 A.M.– 5:56 A.M.	19.7	0.084	0.16
Apr. 12	2:22 A.M.– 6:30 P.M.	16.1	0.044	0.11
Apr. 13–14	8:22 A.M.– 9:44 A.M.	25.1	0.079	0.21
Apr. 14–15	10:32 P.M.–11:55 A.M.	13.4	0.071	0.18
Apr. 16–17	12:11 A.M.– 1:54 A.M.	25.7	0.064	0.13
Apr. 17–18	2:17 P.M.– 4:57 P.M.	26.7	0.087	0.22
Apr. 18–19	6:33 P.M.– 1:23 P.M.	18.7	0.048	0.10
Apr. 23	12:49 A.M.– 6:36 P.M.	17.6	0.051	0.10
Apr. 28–29	9:41 P.M.– 4:31 P.M.	18.7	0.051	0.11
May 2	1:42 A.M.–12:24 P.M.	10.7	0.069	0.12
May 3	2:16 A.M.– 3:47 P.M.	13.3	0.050	0.11
May 3– 4	6:30 P.M.– 3:31 A.M.	9.1	0.142	0.17
May 4– 5	10:31 P.M.– 1:27 P.M.	14.9	0.066	0.22
May 7– 8	5:31 P.M.– 1:31 A.M.	8.0	0.131	0.28
May 9	6:25 A.M.– 6:09 P.M.	11.7	0.053	0.11
May 11–12	5:55 P.M.– 2:50 P.M.	20.9	0.095	0.33
May 12–13	4:58 P.M.– 9:15 P.M.	28.3	0.073	0.17
May 13–14	10:18 P.M.– 2:03 P.M.	15.8	0.070	0.12
May 15	10:50 A.M.– 4:10 P.M.	5.3	0.065	0.10
May 19	10:16 A.M.– 7:20 P.M.	9.1	0.053	0.10
May 22	4:21 A.M.– 5:09 P.M.	12.8	0.075	0.18
May 25	1:06 A.M.– 8:02 A.M.	6.9	0.055	0.10
May 25–26	6:11 P.M.– 3:01 P.M.	20.8	0.053	0.10
May 26–27	8:21 P.M.– 8:05 A.M.	11.7	0.043	0.10
May 28	12:37 A.M.– 9:41 A.M.	9.1	0.055	0.17
May 30	12:05 A.M.–11:49 A.M.	11.7	0.054	0.11
June 1	3:38 A.M.– 2:47 P.M.	11.2	0.064	0.15
June 8– 9	10:20 P.M.– 1:35 P.M.	15.2	0.080	0.20
June 9–10	6:23 P.M.– 1:32 P.M.	19.2	0.060	0.12
June 18	2:49 A.M.– 8:24 P.M.	17.6	0.054	0.11
June 21	1:12 A.M.– 4:40 P.M.	15.5	0.053	0.11
June 30	1:59 A.M.–10:34 A.M.	8.6	0.036	0.15
July 4– 5	9:01 P.M.–10:53 A.M.	13.8	0.083	0.34
July 6– 7	7:41 A.M.–11:25 A.M.	27.7	0.062	0.18
July 15–16	5:32 P.M.– 1:16 P.M.	19.7	0.056	0.21
July 22	4:46 A.M.– 3:58 P.M.	11.2	0.088	0.33
July 22–23	11:26 P.M.– 5:02 P.M.	17.6	0.109	0.23
July 23–24	9:50 P.M.–11:40 A.M.	13.8	0.097	0.16
July 24–25	9:48 P.M.– 4:12 A.M.	6.4	0.053	0.11
July 25	7:56 A.M.– 2:20 P.M.	6.4	0.105	0.17
July 25–26	9:16 P.M.– 3:02 P.M.	17.8	0.070	0.14
Aug. 4	5:36 A.M.– 4:20 P.M.	10.7	0.054	0.10
Aug. 4– 5	9:08 P.M.– 3:48 P.M.	18.7	0.053	0.10
Aug. 5– 6	8:04 P.M.– 2:00 P.M.	17.6	0.085	0.21

TABLE II (*Continued*)

Aug. 9	6:32 A.M.– 5:44 P.M.	11.2	0.102	0.22
Aug. 9–10	10:00 P.M.– 2:32 P.M.	16.5	0.099	0.17
Aug. 12	5:53 A.M.– 2:57 P.M.	9.1	0.071	0.21
Aug. 12–13	9:53 P.M.– 2:09 A.M.	4.3	0.065	0.11
Aug. 15–16	8:48 P.M.– 3:44 A.M.	6.9	0.080	0.21
Aug. 16–17	7:38 P.M.– 3:38 A.M.	8.0	0.071	0.15
Aug. 19–20	11:57 P.M.– 3:26 P.M.	15.5	0.053	0.16
Aug. 20–21	6:06 P.M.–11:44 A.M.	17.6	0.070	0.19
Aug. 27	6:14 A.M.– 3:50 P.M.	9.6	0.078	0.15
Aug. 29	3:08 A.M.– 7:23 A.M.	4.3	0.084	0.16
Sept. 6– 7	11:02 P.M.–12:22 P.M.	13.3	0.061	0.30
Sept. 10	12:38 A.M.–11:18 A.M.	10.6	0.097	0.27
Sept. 14–15	11:24 P.M.– 1:50 P.M.	14.4	0.132	0.30
Sept. 16–17	10:22 P.M.–10:38 A.M.	12.2	0.139	0.35
Sept. 18–19	11:58 P.M.–10:38 A.M.	10.6	0.099	0.18
Sept. 23	1:32 A.M.– 7:40 P.M.	18.1	0.117	0.28
Sept. 23–24	8:12 P.M.– 2:20 P.M.	18.3	0.048	0.16
Sept. 24–25	8:44 P.M.– 3:16 A.M.	6.5	0.090	0.28
Sept. 25–26	7:13 P.M.– 1:21 P.M.	18.1	0.057	0.16
Oct. 17–18	1:48 P.M.– 9:36 A.M.	20.2	0.073	0.12
Oct. 18–19	4:32 P.M.– 2:34 P.M.	21.8	0.056	0.11
Oct. 21–22	2:28 P.M.–12:47 P.M.	22.4	0.094	0.19
Oct. 25–26	4:21 P.M.–10:29 A.M.	18.1	0.084	0.29

Determinations for the period October 1–15 are not included in this report because of possible occasional contamination from a nearby experimental fumigation with animals at a concentration of 63 p.p.m. of sulphur dioxide.

TABLE III

SUMMARY OF RELATIONSHIPS BETWEEN CONCENTRATIONS IN P.P.M. OF SULPHUR DIOXIDE IN THE ATMOSPHERE AND IN THE AIR OF A SMALL GREENHOUSE

	Av. con-centration	Av. of concs.-atmos. concs. above zero	Av. of concs.-atmos. concs. above 0.10	Av. of concs.-atmos. concs. above 0.20	Maximum concen-tration	Minimum concen-tration
	Ventilators partly open 236 concurrent 32-minute runs					
Atmosphere	0.042	0.062	0.139	0.23	0.26	0
Greenhouse	0.039	0.057	0.129	0.21	0.26	0
Greenhouse / Atmosphere	93%	92%	93%	91%	100%	
	Ventilators completely closed 509 concurrent 32-minute runs					
Atmosphere	0.044	0.057	0.161	0.283	0.33	0
Greenhouse	0.031	0.039	0.097	0.166	0.20	0
Greenhouse / Atmosphere	71%	68%	60%	59%	61%	

SOURCES OF THE SULPHUR DIOXIDE

The Boyce Thompson Institute is located in a lightly populated sub-urban residential district on the outskirts of an industrial area. Hourly correlation of SO_2 concentrations with wind direction indicates that the

sulphur dioxide comes largely from New York City (15.4 miles SSW to Times Square which marks the approximate center of the metropolitan area). Some of the gas comes from industrial and domestic heating plants in Yonkers (2.7 miles S), and some small amounts of the gas come from industrial plants in Hastings (1.8 miles NNE) and from the Boyce Thompson Institute heating plant (stacks 76 feet high, 100 feet NNE of sampling station). The Boyce Thompson plant is the only possible local source of the gas, and the fact that only low concentrations of short duration have been recorded when the wind is from the northern sector, NE to NW, indicates that this local source is of minor importance. (See Fig. 1.)

Sulphur dioxide in city atmospheres comes largely from the burning of coal. According to estimates of the United States Department of Commerce as contained in a letter from that department dated August 12, 1936 and signed by Paul L. Hopper and J. J. W. Palmer, the annual consumption of bituminous coal in New York City is eleven million tons. Bituminous coal of the type entering the New York market would run perhaps 1.5 per cent sulphur so that by the consumption of eleven million tons, 330,000 tons of SO_2 are thrown into the atmosphere. There also enter the New York market some eight million tons of anthracite coal which would run about 1 per cent sulphur, throwing into the air an additional 160,000 tons of SO_2. If fuel oil and gasoline consumption are also considered, it seems likely that the amount of sulphur dioxide thrown into the air of New York City is on the order of 2,000 tons per day. Estimates (6) based on a recent WPA Air Pollution Survey show that, exclusive of the discharge from internal combustion engines, the amount of sulphur dioxide discharged into the atmosphere of New York City during 1934 averaged 1989 tons per day.

CONDITION OF VEGETATION

It is interesting to note that the presence of sulphur dioxide in the air at Boyce Thompson Institute in measurable concentrations was not suspected until the present study was undertaken.

The many plants grown throughout the year in the Institute greenhouses, and the vegetation in and about the Institute grounds, are considered comparable to greenhouse plants and vegetation growing in sulphur dioxide-free areas. Located near the Institute are many well known gardens.

The evidence seems to indicate that the low concentrations of SO_2 prevailing in the atmosphere at the Institute have no harmful effect on the plants, despite the fact that the high yearly rainfall (45 inches) and relatively high average humidity (60 per cent) would tend to make the plants more susceptible to SO_2 than plants growing in drier areas.
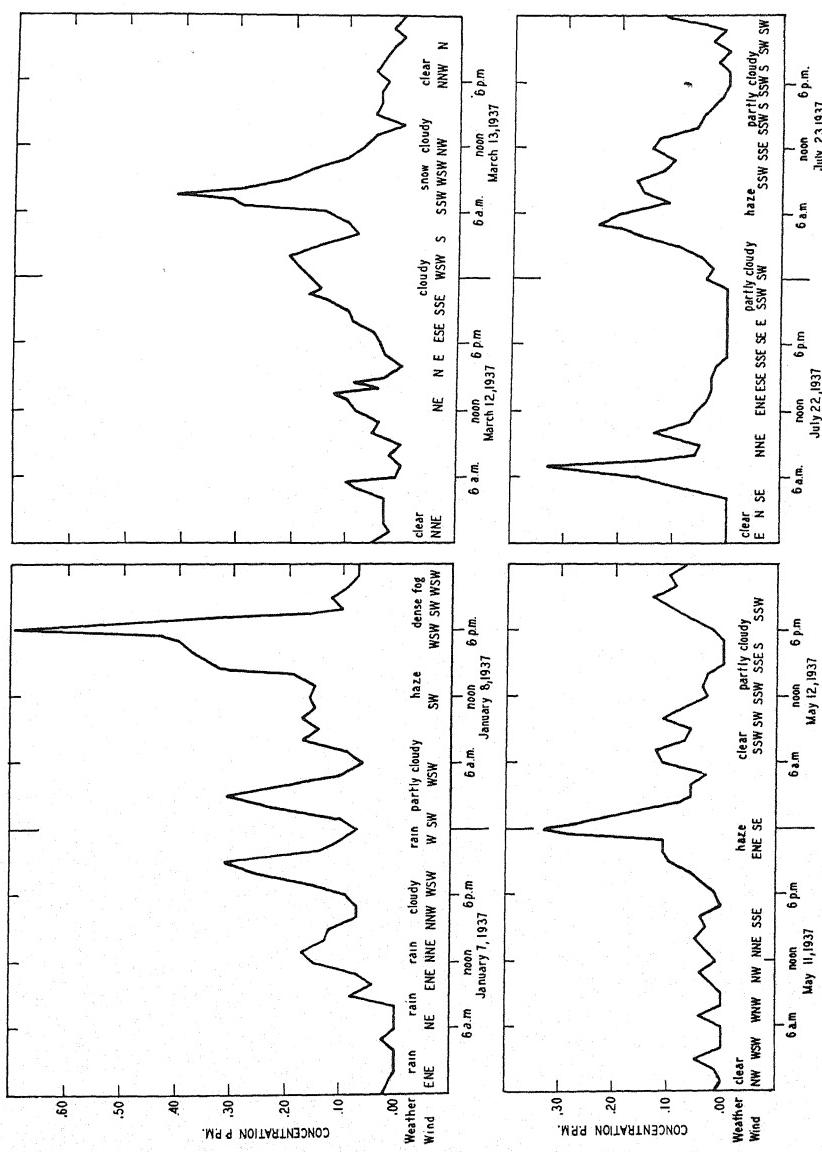
FIGURE 1. Variations in concentrations of sulphur dioxide in the atmosphere at different times of the year.

SUMMARY

The sulphur dioxide content of the prevailing atmosphere at Boyce Thompson Institute was determined continuously from November 1, 1936 to November 1, 1937, with minor interruptions.

For the year period, the average reading including zero readings was 0.033 p.p.m. Maximum concentration recorded was 0.75 p.p.m. The gas was present, in concentrations of 0.01 p.p.m. and over, 62.2 per cent of the time.

Correlation of SO_2 concentrations with the wind direction indicates that the sulphur dioxide comes largely from New York City (15.4 miles SSW to Times Square which marks the approximate center of the metropolitan area).

A study of the relationships between concentrations of sulphur dioxide in the atmosphere and in the air of a greenhouse shows that greenhouse concentrations are approximately 90 per cent of atmospheric when ventilators are partly open, 60 per cent when ventilators are closed.

The fact that the many plants grown throughout the year in the Institute greenhouses are considered comparable to plants grown in areas where there is no sulphur dioxide, is an indication that exposure to sulphur dioxide in the prevailing concentrations and durations has no unfavorable effect on plant life.

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EFFECT OF LOW CONCENTRATIONS OF SULPHUR DIOXIDE ON YIELD OF ALFALFA AND CRUCIFERAE

CARL SETTERSTROM, P. W. ZIMMERMAN, AND WILLIAM CROCKER

Sulphur dioxide, as one of the gaseous products of the burning of coal and of some industrial operations, is released into the atmosphere of industrial centers in large quantities. Recent studies by the Air Hygiene Foundation (11), the National Research Council of Canada (7), the United States Department of Agriculture (23) and the Boyce Thompson Institute (16) reveal that measurable concentrations of sulphur dioxide prevail over large areas surrounding these industrial centers.

The injurious effects of sulphur dioxide on plants have been discussed at length and in detail over a period dating back to the work of Stöckhardt (18) in 1848. Investigators are in general agreement that concentrations of the gas on the order of 1 p.p.m. will cause typical foliar markings on more sensitive species in a few hours under favorable conditions, and that higher concentrations may cause complete defoliation and death.

Investigators do not agree, however, on the effects of long exposure of plants to low concentrations of the gas which do not produce visible markings; concentrations such as prevail over the larger part of the areas subjected to fumes of sulphur dioxide.

INVISIBLE INJURY

Proponents (4, 6, 19, 24) of the "invisible injury" theory maintain that the gas is harmful in all concentrations and under all circumstances; that it always has an adverse effect on yield. Some opponents (5, 20) of this theory believe that the gas does not reduce yield unless it causes markings. Other opponents (13) maintain that exposure to low non-marking concentrations stimulates growth.

The implications of the "invisible injury theory" are of considerable importance to industrial as well as to agricultural interests. As Swain (20) has emphasized, if this theory be correct then any industrial operation which contributes sulphur dioxide to the atmosphere is not merely a potential but an actual agent of injury, and the subject of injunctive relief as a nuisance in the eyes of the law.

Until recently, however, no convincing experimental evidence on the effects of low non-marking concentrations had been presented. Wieler (24), Stoklasa (19), Bredemann (1, 4, p. 285), Janson (6), and other "invisible injury" supporters based their arguments on field observations, which are at best beset by numerous uncontrolled variables, and on the assumption that SO_2 interferes with the assimilative processes.

O'Gara (13), whose experimental work marked a great advance in the study of the effects of sulphur dioxide, declared that in all the cases where he treated plants he found an actual stimulation of growth until he reached a point where visible injury was produced. Data of Hill and Thomas (5) indicate that sulphur dioxide has no effect on yield of alfalfa unless it produces visible effect. But these studies of O'Gara and of Hill and Thomas were concerned with the causes and consequences of visible injury rather than with the effects of non-marking concentrations.

Within the past year, however, three noteworthy studies on the effects of low non-marking concentrations of sulphur dioxide have appeared in the literature. Swain and Johnson (21) report that exposure to low concentrations for considerable periods of time had no measurable effect on the yield of wheat grown in nutrient solutions under ideal conditions of light, temperature, and humidity.

Thomas and Hill (22), as a result of intensive studies of the relation of sulphur dioxide to photosynthesis and respiration of alfalfa, present convincing evidence that in fumigations which did not produce visible injury there was no "invisible injury" either to protoplasm or other plant structure. Katz and Ledingham (8, 9) describe assimilation studies substantiating the conclusions of Thomas and Hill, and also present considerable quantitative data on gross yield of alfalfa plots fumigated with sulphur dioxide as compared with control plots. These data show that prolonged sulphur dioxide treatment without leaf injury had no significant effect on yield.

None of these studies, however, attempted to determine the "invisible" effects of sulphur dioxide under conditions unfavorable to plant growth such as are frequently encountered in actual practice.

The experiments conducted at Boyce Thompson Institute, and reported in the present paper, were designed to study the effects of low concentrations on plants grown under different conditions. Water supply, nutrient supply, sulphur content of nutrient supply, and age of plants were varied to simulate many of the conditions encountered in the field.

DESIGN OF EXPERIMENTS

The design of the fumigation experiments for statistical treatment was in accordance with the suggestions of W. J. Youden of this Institute. The following discussion of the lay-out of the experiments and of the statistical treatment of the yield data has been adapted from an unpublished memorandum written by Dr. Youden and from an introduction to a paper by Fisher and Wishart (3).

The experiments are of a factorial type. A number of factors (such as SO_2, H_2O, nutrient supply, and sulphur content of nutrient supply) were studied simultaneously. This scheme permits the mobilization of the

entire experiment back of each contrast (such as SO_2 vs. check). A great improvement in precision results from the large number of plants involved and the consequent minimizing of chance deviations of individual plants. Great dependence may be placed on the results because the contrast (such as SO_2 vs. check) rests on a broad basis of varied conditions (such as different H_2O, nutrient and S levels) rather than on some arbitrary set running through the whole group. Factorial experiments of this nature are especially adapted to determining whether the effect of some factor is more pronounced under some conditions than others. More reliance may be placed upon the results than if separate experiments were performed because in the latter case a second lot of plants introduces a new variable if the plants are grown in a different space or at a different time.

The interpretation of such complex experiments follows a logical and systematic contrasting of pairs (or groups) of plants, the numbers of each pair having been given identical treatment save in respect to the factor under investigation. The same data regrouped into different pairs bring out some other factor and so in turn each condition may be investigated. The results, of what in using the older methods would have constituted several separate experiments, may be freely intercompared. This is an advantage often sacrificed when separate experiments are involved since it is difficult to give the several experiments the same starting material, care, temperature, etc.

The yield data obtained from these experiments have been subjected to statistical analysis by the method of the analysis of variance developed by Fisher (2). The analysis of variance is recognized as a standard method of evaluating results. The procedure is impartial and the final decision rests on the verdict of the tables published in statistical works. All the data are used instead of some particular case which happens to illustrate the point at hand and which, being an isolated case, may have been an example of extreme variation occurring naturally.

Statisticians have established with exactness what the required magnitudes of the ratio

$$\frac{(\text{difference arising from an imposed factor})^2}{(\text{difference between duplicates})^2}$$

must be, for experiments involving various numbers of individuals, if significance is to be attached to the result. It is true that the statistician expresses his results in terms of probabilities and not as certainties; that is, he finds that a certain difference between two means could have occurred by chance, not more than, say, once in 20 trials. But by fixing an appropriate standard of significance which is widely recognized he distinguishes between the results which should be ignored and those which

should form the basis of his conclusions. In the analyses of variance presented in this paper, differences which could have occurred by chance not more than once in 20 trials are termed significant, differences which could have occurred by chance not more than once in 100 trials are termed highly significant.

APPARATUS

The apparatus used in this study consists of duplicate sets, one for fumigating plants with sulphur dioxide, the other for controls. Each set includes a cabinet of special design and construction in which the plants are placed during the experiment; a variable speed exhauster-blower and an orifice meter for precise control of the volume of air passing through the cabinet; specially designed scrubbers attached to the intake side of the blowers to rid the air of SO_2 before allowing it to enter the system and to effect a partial control of the humidity in the cabinet; a Thomas autometer and Leeds and Northrup conductivity recorder for determining sulphur dioxide content of the air in the cabinet; a Bristol humidigraph for recording the temperature and relative humidity in the cabinet.

Accurate metering of the sulphur dioxide entering the test cabinet is made possible by the use of calibrated capillary flowmeters, a reducing regulator, a sensitive needle valve, and a cylinder containing a special mixture of 5 per cent sulphur dioxide and 95 per cent air.

All the apparatus, except the conductivity recorders, is housed in a small greenhouse which is provided with automatic temperature control. The conductivity recorders are installed in a laboratory of the Institute about 20 feet from the greenhouse.

In every experimental fumigation the concentration of SO_2 in the test cabinet as calculated by readings of the calibrated capillary flowmeter and calibrated orifice meter was in close agreement with the continuous record of the conductivity recorder and with occasional determinations with the iodine method.

In the yield experiments the sets of apparatus were alternately used for fumigation and for control in order to offset any possible advantage of one cabinet over the other.

For a detailed description of the apparatus see a previous paper by Setterstrom and Zimmerman (15).

EXPERIMENTAL PROCEDURE

In order to obtain material with varying sulphur content the plants used in these experiments were grown from seed in 5-inch pots containing sand, and were watered twice weekly with nutrient solutions containing varying amounts of sulphur. In preparing these nutrient solutions the following aqueous stock solutions were used:

5% Potassium nitrate (KNO_3)
5% Potassium acid phosphate (KH_2PO_4)
10% Calcium nitrate $Ca(NO_3)_2$
5% Magnesium sulphate ($MgSO_4$)
3.95% Magnesium chloride ($MgCl_2$)
5% Potassium sulphate (K_2SO_4)
1% Ferric chloride ($FeCl_3$)

To prepare a liter of nutrient solution, the following were added to 500 cc. of distilled water and the resultant solution made up to a liter:

1. One cc. ferric chloride solution and twenty-five cc. each of the potassium nitrate, potassium acid phosphate, and calcium nitrate stock solutions.
2. The volumes of magnesium chloride, magnesium sulphate, or potassium sulphate stock solution listed below for the particular nutrient solution.

Experiments No. 1 and 2

Solution number	Added ingredients	% S
1	50 cc. K_2SO_4	0.079
	25 cc. $MgSO_4$	
2	25 cc. K_2SO_4	0.056
	25 cc. $MgSO_4$	
3*	25 cc. $MgSO_4$	0.033
4	25 cc. $MgCl_2$	0.000
5	20 cc. $MgSO_4$	0.026
	5 cc. $MgCl_2$	
6	15 cc. $MgSO_4$	0.020
	10 cc. $MgCl_2$	
7	10 cc. $MgSO_4$	0.013
	15 cc. $MgCl_2$	
8	5 cc. $MgSO_4$	0.007
	20 cc. $MgCl_2$	

* "Normal" supply of sulphur.

Experiments No. 3, 4, and 5

S+	54.3 cc. K_2SO_4, 25 cc. $MgSO_4$	0.083
S*	25 cc. $MgSO_4$	0.033
S−	10 cc. $MgSO_4$, 15 cc. $MgCl_2$	0.013

* "Normal" supply of sulphur.

When ready for fumigation the pots containing the plants were, in every case, assigned to the fumigation or to the control cabinet solely by chance—either by tossing a coin or by drawing lots.

Pertinent data regarding concentration, temperature, humidity; detailed yield data; appropriate tables; and the analysis of variance of each experiment are included under the heading "Experimental Results."

Hourly figures for solar and sky radiation in gram-calories per sq. cm. of horizontal surface and other pertinent meteorological data are available in reports of the New York Meteorological Observatory (12).

The sulphur content of leaves and stems of fumigated and control plants were determined for experiments No. 4 and 5 and are included in the results of those experiments.

Concentrations of 0.10, 0.15, and 0.20 parts of sulphur dioxide per million parts of air by volume were used because these concentrations approximate the average of all concentrations above zero which prevail over the greater part of agricultural areas near industrial centers.

Alfalfa (*Medicago sativa* L. var. Grimm) was selected as the principal test plant because of its sensitivity to sulphur dioxide, and because the alfalfa crop is of considerable economic value in several of the large agricultural areas which are subjected to sulphur dioxide fumes.

EXPERIMENTAL RESULTS
EXPERIMENT NO. 1 (0.104 P.P.M. SO₂ FOR 650 HOURS)

It was felt that the amount of available sulphur in the soil might condition the growth response of a plant to sulphur dioxide. Investigators who found stimulation with SO_2 treatment may have grown their plants in sulphur-deficient soil, and those who found no effect on yield may have used plants with an adequate sulphur supply. Furthermore, plants growing in soil containing an excess of sulphur may actually have been retarded on treatment with sulphur dioxide as would be the case with an over-supply of fertilizer.

To test these hypotheses, plants were grown in nutrient solutions with sulphur content varying up to 0.086 per cent, or three times what was considered "normal" or optimum for best growth, and then were fumigated with 0.104 p.p.m. of SO_2 for 650.46 hours.

Grimm alfalfa was selected as the test plant. The fumigation took place in late December and early January when the days were short, light intensity was low, and the plants were growing poorly.

The concentration, temperature, and humidity data are summarized in Table I. In referring to concentration, the heading "No. Observations" designates the number of recorder readings, each reading extending over the 32-minute sampling period. Humidigraph recordings of temperature and humidity were continuous, but in this and other long fumigations hourly readings were averaged to give the figure for "Average." Yield data are given in Table II.

TABLE I

SO₂ Concentration, Temperature, and Humidity Data for the Experimental Fumigations

	Experiment 1 Dec. 16 to Jan. 12 (650 hrs.)				Experiment 2 Feb. 15 to Mar. 4 (402 hrs.)				Experiment 3 Mar. 5 to Mar. 10 (117 hrs.)				Experiment 4 Mar. 11 to Apr. 5 (600 hrs.)				Experiment 5 Apr. 7 to May 2 (600 hrs.)			
	Av.	Max.	Min.	No. obs.	Av.	Max.	Min.	No. obs.	Av.	Max.	Min.	No. obs.	Av.	Max.	Min.	No. obs.	Av.	Max.	Min.	No. obs.
SO₂ conc. (p.p.m.)																				
Fumigation cabinet																				
Theoret. inlet	.10				.15				.20				.10				.10			
Recorded inlet	.104	.16	.05	1149	.156	.20	.08	756	.206	.23	.17	218	.105	.14	.05	1100	.105	.14	.05	1120
Recorded outlet	.103	.16	.05	1173	.152	.20	.08	615	.200	.22	.15	218	.097	.13	.04	960	.105	.14	.05	1120
Control cabinet																				
Recorded inlet	.005	.05	.00	1098	.002	.03	0.00	609	.000	.01	.00	218	.003	.02	0.00	1080	.001	0.03	0.00	1113
Atmospheric	.053	.69	.00	1141	.042	.75	0.00	743	.029	.17	.00	218	.028	.41	0.00	1119	.028	0.22	0.00	1120
Temperature (°F.)				Continuous				Continuous				Continuous				Continuous				Continuous
Fumigation cabinet	69.7	85	57		69.9	87	60		67.1	86	60		67.2	104	59		65.6	87	57	
Control cabinet	72.0	97	57		73.0	86	65		68.8	80	63		66.1	97	58		68.6	96	60	
Relative humidity (%)																				
Fumigation cabinet	61.7	79	26		61.9	77	30		64.7	75	35		65.0	79	25		64.5	88	28	
Control cabinet	61.3	75	23		65.2	76	40		63.4	70	49		63.9	71	26		61.3	81	31	

Note: The volume of air passing through the cabinets was 123 cubic feet per minute in exp. 1 and 142 cubic feet per minute in the four other experiments.

The northernmost of the two cabinets was used as the fumigation cabinet in experiments 1 and 5 and as the control cabinet in experiments 2, 3, and 4.

TABLE II

EFFECT OF SO_2 ON YIELD OF ALFALFA†. EXPERIMENT NO. 1. 0.104 P.P.M. FOR 650 HOURS

Grams of S per 100 cc. of nutrient solution	Green weights—g. per pot		Dry weights—g. per pot	
	Treated (SO_2)	Control	Treated (SO_2)	Control
0.079	10.2 11.6	10.1 13.6	2.09 1.80	1.92 2.75
0.056	15.8 18.0	15.7 17.8	2.61 2.81	3.44 2.63
0.033	12.4 18.5	11.6 16.2	2.19 2.67	2.64 2.74
0.026	12.5 19.3	8.6 12.5	2.02 3.03	1.81 2.39
0.020	13.0 13.2	10.6 10.7	2.15 2.28	2.26 2.10
0.013	10.8 11.4	8.1 10.4	1.78 1.86	1.40 1.75
0.007	7.6 8.9	5.1 6.3	1.46 1.47	1.00 1.07
0.000	7.0 11.5	9.7 10.1	1.95 1.11	2.01 1.75
Average	12.6	11.1	2.08	2.10

† Alfalfa planted November 16, 1936; fumigated December 16, 1936 to January 12, 1937; and harvested February 18, 1937.

ANALYSIS OF VARIANCE

Source of variation	Degrees of freedom	Green weights			Dry weights		
		Sum of squares	Mean square	F††	Sum of squares	Mean square	F††
SO_2	1	18.91	18.91	3.5	0.0045	0.0045	
Sulphur conc.	7	266.37	38.05	7.1**	7.6185	1.0883	8.6**
SO_2 vs. S	7	29.39	4.20		0.8701	0.1243	
Duplicates	16	86.26	5.39		2.0078	0.1255	
Total	31	400.93			10.5009		

** Highly significant.

†† F is a symbol used by Snedecor (17) to designate the ratio of the larger mean square to the smaller. The significance of F is determined by consulting Snedecor's Table XXXV.

Examination of the analyses of variance of green and of dry weights reveals that the increase in weight of the sulphur dioxide treated plants is not significant. The increments of sulphur concentration were not large enough to bring about significant yield differences referable to sulphur content of nutrient supply, and the sulphur dioxide effect on yield is not significantly different at the varying levels of sulphur supply.

TABLE III

EFFECT OF SO₂ ON YIELD OF ALFALFA.† EXPERIMENT NO. 2. 0.154 P.P.M. FOR 402 HOURS

Grams of S per 100 cc. of nutrient solution	Green weights—g. per pot				Dry weights—g. per pot			
	Planted February 5, 1937		Planted December 18, 1936		Planted February 5, 1937		Planted December 18, 1936	
	SO₂	Control	SO₂	Control	SO₂	Control	SO₂	Control
0.079	17.4	16.8	16.9	21.7	2.86	2.69	2.97	4.15
	17.1	16.6	22.5	19.2	2.85	2.64	3.87	3.43
0.056	21.1	20.4	35.4	27.0	3.36	2.93	6.59	4.94
	20.0	16.9	28.8	17.1	3.10	2.41	5.53	2.85
0.033	20.6	13.1	26.8	14.7	3.02	1.86	4.43	2.55
	20.1	12.6	21.7	19.9	3.27	1.94	3.82	3.55
0.026	22.8	21.2	26.8	25.6	3.34	3.10	4.38	4.51
	16.7	15.3	21.8	18.6	2.68	2.11	3.79	3.12
0.020	22.9	19.9	27.8	33.2	3.50	3.06	4.57	6.15
	19.6	10.1	33.8	17.4	2.82	1.68	5.82	2.98
0.013	23.7	12.9	22.1	14.3	3.26	2.15	3.82	2.54
	6.6	20.7	12.9	16.5	1.10	2.93	2.37	2.91
0.007	22.9	19.9	32.0	19.7	3.35	2.99	4.87	3.20
	17.1	12.9	34.4	17.0	3.00	1.82	5.68	2.77
0.000	18.6	13.8	23.6	29.8	2.83	1.81	4.02	4.84
	21.6	18.7	26.8	26.4	3.09	2.74	4.67	4.67
Average	19.3	16.4	25.9	21.1	2.96	2.43	4.45	3.70

† Alfalfa fumigated from February 15, 1937 to March 4, 1937 and harvested March 25, 1937.

ANALYSIS OF VARIANCE

Source of variation	Degrees of freedom	Green weights			Dry weights		
		Sum of squares	Mean squares	F	Sum of squares	Mean squares	F
SO₂	1	236.39	236.39	11.0**	6.5758	6.5758	11.0**
Age	1	515.29	515.29	23.9**	30.3463	30.3463	50.1**
Sulphur conc.	7	370.54	52.93	2.5*	10.2714	1.4645	2.5*
Age vs. SO₂	1	13.14	13.14		0.2494	0.2494	
Age vs. S	7	141.70	20.24		4.7941	0.6849	
S vs. SO₂	7	186.06	26.58		5.8001	0.8286	
S age SO₂	7	114.69	16.38		2.9180	0.4154	
Duplicates	32	689.07	21.53		19.0877	0.5965	
Totals	63	2266.88			80.0428		

* Significant.
** Highly significant.

EXPERIMENT NO. 2 (0.154 P.P.M. SO$_2$ FOR 402 HOURS)

This experiment was designed to repeat Experiment 1 at a higher concentration and to furnish a study of the growth effect on plant series of two ages. Grimm alfalfa, 10 days old and 59 days old at the beginning of

TABLE IV

EFFECT OF SO$_2$ ON YIELD OF CRUCIFERAE†. EXPERIMENT NO. 3. 0.203 P.P.M. FOR 117 HOURS

			Green weights—g. per pot				Dry weights—g. per pot			
			Ruta-baga	Cab-bage	Tur-nip	Mus-tard	Ruta-baga	Cab-bage	Tur-nip	Mus-tard
Control	Ample water	S+	32.2	56.5	53.4	29.5	2.38	4.46	3.71	2.13
		S	42.2	33.2	115.4	50.5	3.07	2.20	7.90	3.38
		S−	48.1	29.3	39.2	43.7	3.70	2.09	3.21	3.23
	Less water	S+	37.2	31.8	51.4	54.0	2.91	2.23	4.10	4.08
		S	61.2	44.2	84.3	69.6	4.57	2.68	6.49	5.10
		S−	47.2	42.4	66.5	33.6	4.17	3.15	5.60	2.52
Average			44.6	39.5	68.3	46.8	3.47	2.81	5.16	3.40
Treated (SO$_2$)	Ample water	S+	37.5	46.4	65.8	46.2	2.85	3.20	4.87	3.26
		S	69.4	37.5	97.2	75.9	5.26	2.55	7.65	5.12
		S−	44.5	32.1	72.7	32.8	3.44	2.05	5.13	2.33
	Less water	S+	39.7	33.4	65.0	60.2	3.38	2.35	5.71	4.69
		S	71.8	62.8	79.8	65.6	5.53	4.05	5.48	4.43
		S−	31.2	45.8	83.4	35.0	1.98	3.10	6.04	2.40
Average			49.0	43.0	77.3	52.6	3.74	2.88	5.81	3.70

Note: The sulphur concentrations of the nutrient solutions were 0.083, 0.033, and 0.013 g. per 100 cc. of solution and are designated S+, S, and S−, respectively.

† The Cruciferae were planted January 18, 1937; fumigated March 5, 1937 to March 10, 1937, and harvested March 23, 1937.

ANALYSIS OF VARIANCE

Source of variation	Degrees of free-dom	Green weights			Dry weights		
		Sum of squares	Mean square	F	Sum of squares	Mean square	F
SO$_2$	1	380.25	380.25	2.2	1.2352	1.2352	1.1
H$_2$O	1	90.48	90.48		1.1656	1.1656	
Variety	3	6949.73	2316.58	13.1**	46.0466	15.3489	13.7**
Sulphur conc.	2	4453.61	2226.81	12.6**	17.3599	8.6800	7.7*
SO$_2$ vs. S	2	32.74	16.37		0.3326	0.1663	
H$_2$O vs. S	2	45.42	22.71		0.2237	0.1119	
H$_2$O vs. SO$_2$	1	24.81	24.81		0.4447	0.4447	
Var. vs. SO$_2$	3	52.71	17.57		0.5173	0.1727	
Var. vs. H$_2$O	3	124.71	41.57		0.4466	0.1489	
Var. vs. S	6	1492.94	248.82	1.4	8.3423	1.3904	1.2
Error	23	4068.22	176.87		25.7613	1.1201	
Total	47	17715.62			101.8758		

* Significant.
** Highly significant.

the fumigation, was used as the test material. The younger alfalfa was just breaking through the soil surface, the older alfalfa was about four inches high. During the fumigation the plants grew rapidly and were in good condition. Concentration, temperature, and humidity data are summarized in Table I. Yield data are given in Table III.

Examination of the analyses of variance of green and of dry weights reveals that the increase in both green and dry weights of the sulphur dioxide treated plants is highly significant, as is the effect of the age of the plants. Sulphur content of nutrient supply has a significant effect on yield, but the sulphur dioxide effect is not significantly different at the different levels of sulphur supply or at the different plant ages.

TABLE V

CONDITIONS UNDER WHICH ALFALFA WAS GROWN IN EXPERIMENTS 4 AND 5, AND THE EFFECT OF SULPHUR DIOXIDE TREATMENT ON YIELD UNDER THESE CONDITIONS

Experimental conditions	No. of pots in each experiment	% increase in weight of SO$_2$ treated plants over controls*		% decrease in weight of SO$_2$ treated plants under controls*	
		Exp. 4	Exp. 5	Exp. 4	Exp. 5
Excess sulphur in nutrient solution	16			4.3	9.4
Normal sulphur in nutrient solution	16	24.4	15.4		
Deficiency of sulphur in nutrient solution	16	41.5	4.6		
Full nutrient supply	24	31.9	9.6		
Deficient nutrient supply (poor plants)	24	9.2			9.5
Ample water	24	24.6			7.0
Less water	24	19.0	27.5		
Full nutrient supply—excess sulphur	8	4.8			6.2
Full nutrient supply—normal sulphur	8	25.1	34.0		
Full nutrient supply—deficiency of sulphur	8	56.9	0.1		
Deficient nutrient supply—excess sulphur	8			10.6	6.1
Deficient nutrient supply—normal sulphur	8	23.1			38.6
Deficient nutrient supply—deficiency of sulphur	8	17.0	24.0		
Ample water—excess sulphur	8			12.1	13.6
Ample water—normal sulphur	8	12.1			0.5
Ample water—deficiency of sulphur	8	92.0			7.3
Less water—excess sulphur	8	8.1	7.6		
Less water—normal sulphur	8	41.0	50.4		
Less water—deficiency of sulphur	8	3.9	24.7		
Full nutrient supply—ample water	12	24.2			2.5
Full nutrient supply—less water	12	40.7	32.0		
Deficient nutrient supply—ample water	12	24.1			20.4
Deficient nutrient supply—less water	12		12.8	6.7	

* Because of the inherent variability of biological material, it is reasonable to discount the lower percentages as natural variations and indications of no effect on yield.

EXPERIMENT NO. 3 (0.203 P.P.M. SO₂ FOR 117 HOURS)

In this experiment certain high sulphur-containing Cruciferae (all of the genus *Brassica*) were used as test material, namely cabbage (*B. oleracea* var. *capitata* L.—Early Jersey Wakefield), rutabaga (*B. napobrassica* Mill. var. Purple-Top Yellow), mustard (*Brassica* sp. var. Fordhook Fancy), and turnip (*B. rapa* L. var. Snowball). The plants were 46 days old at the beginning of the fumigation and in good condition. The concentration, temperature, and humidity data are summarized in Table I. Yield data are given in Table IV.

Examination of the analyses of variance of green and of dry weights reveals that although there is consistent increase in yield of each variety under every condition when treated with SO₂ the increase is not large enough to be significant. Sulphur content of nutrient supply and variety of Cruciferae both had a highly significant effect on yield.

EXPERIMENT NO. 4 (0.101 P.P.M. SO₂ FOR 600 HOURS)

In order to determine whether under certain conditions low concentrations of sulphur dioxide cause significant decreases in yield of alfalfa, plants were grown under many different experimental conditions and the effect of SO₂ under these various conditions was determined. The treatments and results are summarized in Table V. The concentration, temperature, and humidity data are summarized in Table I. Detailed yield data are given in Table VI.

Examination of the analyses of variance of green and of dry weights reveals that the increase in green weights on treatment with sulphur dioxide is significant, although the increase in dry weights is not significant. The supply of nutrients, and sulphur content of nutrient supply have a highly significant effect on yield. The effect of nutrient supply is significantly different at the various levels of sulphur concentration. The effect of water supply is not significant.

EXPERIMENT NO. 5 (0.105 P.P.M. SO₂ FOR 600 HOURS)

This experiment was designed to repeat experiment 4 with older plants. The conditions under which the alfalfa was grown and the effect on yield under these conditions are summarized in Table V. The concentration, temperature, and humidity data are summarized in Table I. Detailed yield data are given in Table VII.

Examination of the analyses of variance of green and of dry weights reveals that although the increase in weight of all sulphur dioxide treated plants is not significant, the increase in green weight of the plants which received less than the normal supply of water is of significance. The effect of supply of nutrients and of the water supply is of great significance. The effect of nutrient supply is significantly different at the various levels of

TABLE VI

EFFECT OF SO_2 ON YIELD OF ALFALFA.† EXPERIMENT NO. 4. 0.101 P.P.M. FOR 600 HOURS

			Green weights— g. per pot			Dry weights— g. per pot		
			S+	S	S−	S+	S	S−
Control	Ample water	Full nutrient supply	8.2	21.1	13.7	1.12	2.71	1.89
			7.4	17.7	2.5	1.12	2.48	2.43
		Deficient nutrient supply	7.6	7.5	9.5	0.96	1.07	1.06
			14.0	9.7	2.7	1.70	1.35	0.31
	Less water	Full nutrient supply	5.5	7.0	11.1	0.90	1.01	1.70
			3.6	15.1	15.4	0.58	2.40	2.35
		Deficient nutrient supply	6.3	10.0	7.0	0.93	1.24	0.98
			7.7	8.9	7.8	0.89	1.07	0.99
		Average	7.5	12.1	8.7	1.03	1.67	1.46
Treated (SO₂)	Ample water	Full nutrient supply	7.9	18.6	13.5	1.13	2.50	1.80
			6.6	23.1	18.2	1.19	3.16	2.36
		Deficient nutrient supply	11.2	12.1	8.5	1.60	1.44	0.93
			7.0	9.1	15.5	1.24	1.28	1.91
	Less water	Full nutrient supply	5.5	11.4	19.6	0.87	1.79	2.89
			5.9	23.1	15.7	0.95	3.69	2.38
		Deficient nutrient supply	7.5	13.1	3.1	1.13	1.58	0.38
			6.1	10.2	4.5	0.86	1.34	0.59
		Average	7.2	15.1	12.3	1.12	2.10	1.66

Note: The sulphur concentrations of the nutrient solutions were 0.083, 0.033, and 0.013 g. per 100 cc. of solution and are designated S+, S, and S−, respectively.

† Alfalfa planted March 1, 1937; fumigated March 11, 1937 to April 5, 1937; and harvested April 8, 1937.

ANALYSIS OF VARIANCE

Source of variation	Degrees of freedom	Green weights			Dry weights		
		Sum of squares	Mean square	F	Sum of squares	Mean square	F
SO₂	1	52.08	52.08	4.4*	0.6888	0.6888	3.3
Sulphur	2	310.64	155.32	13.1**	5.3041	2.6521	12.8**
Nutrient conc.	1	171.76	171.76	14.5**	7.1843	7.1843	34.6**
H₂O	1	36.40	36.40		0.5742	0.5742	2.8
SO₂ vs. S	2	35.65	17.83		0.2385	0.1193	
H₂O vs. S	2	18.55	9.28		0.1998	0.0999	
H₂O vs. SO₂	1	1.85	1.85		0.0238	0.0238	
Nu. vs. SO₂	1	20.81	20.81		0.1092	0.1092	
Nu. vs. H₂O	1	0.15	0.15		0.0050	0.0050	
Nu. vs. S	2	208.59	104.30	8.8**	5.5233	2.7617	13.3**
S SO₂ Nu.	2	7.99	4.00		0.0789	0.0395	
S Nu. H₂O	2	96.19	48.10	4.0*	0.5475	0.2738	
H₂O SO₂ Nu.	1	9.88	9.88		0.6099	0.6099	2.9
H₂O SO₂ S	2	48.12	24.06		0.2824	0.1412	
Error	26	307.48	11.83		5.3951	0.2075	
Total	47	1326.14			26.7648		

* Significant.
** Highly significant.

TABLE VII

EFFECT OF SO$_2$ ON YIELD OF ALFALFA.† EXPERIMENT NO. 5. 0.105 P.P.M. FOR 600 HOURS

			Green weights—g. per pot			Dry weights—g. per pot		
			S+	S	S−	S+	S	S−
Control	Ample water	Full nutrient supply	38.4 41.9	40.3 45.5	37.7 49.9	6.38 6.51	6.26 7.58	3.93 8.08
		Deficient nutrient supply	9.4 16.6	11.4 21.8	4.4 17.1	1.37 2.22	1.69 2.86	0.67 2.48
	Less water	Full nutrient supply	22.2 16.0	17.9 27.3	26.3 26.5	3.52 2.33	2.83 4.43	3.88 3.98
		Deficient nutrient supply	11.7 6.9	6.3 4.5	3.4 8.7	1.49 0.88	0.86 0.62	0.43 1.07
		Average	20.4	21.9	21.8	3.09	3.39	3.07
Treated (SO$_2$)	Ample water	Full nutrient supply	35.3 33.3	47.2 55.6	29.9 45.8	5.28 5.79	8.70 7.65	7.40 4.69
		Deficient nutrient supply	11.9 11.3	5.9 9.6	14.1 11.4	1.57 1.31	1.32 0.81	1.37 2.00
	Less water	Full nutrient supply	24.5 17.9	36.4 36.4	34.9 29.9	2.93 4.16	5.86 5.85	4.66 5.49
		Deficient nutrient supply	6.4 12.3	7.6 3.9	7.3 8.8	1.53 0.76	0.52 0.96	1.19 0.93
		Average	19.1	25.3	22.8	2.92	3.96	3.47

Note: The sulphur concentrations of the nutrient solutions were 0.083, 0.033, and 0.013 g. per 100 cc. of solution and are designated S+, S, and S−, respectively.

† Alfalfa planted March 15, 1937; fumigated April 7, 1937 to May 2, 1937; harvested May 4, 1937.

ANALYSIS OF VARIANCE

Source of variation	Degrees of free-dom	Green weights			Dry weights		
		Sum of squares	Mean square	F	Sum of squares	Mean square	F
SO$_2$	1	13.54	13.54		0.8480	0.8480	1.1
Sulphur	2	122.18	61.09	2.5	3.6814	1.8407	2.3
Nutrient conc.	1	7112.63	7112.63	294.6**	197.0731	197.0731	242.8**
H$_2$O	1	1217.05	1217.05	50.4**	28.1521	28.1521	34.8**
SO$_2$ vs. S	2	44.67	22.34		1.0765	0.5383	
H$_2$O vs. S	2	33.01	16.51		1.1374	0.5687	
H$_2$O vs. SO$_2$	1	107.12	107.12	4.5*	2.3674	2.3674	2.9
Nu. vs. SO$_2$	1	49.83	49.83	2.1	2.5762	2.5762	3.2
Nu. vs. H$_2$O	1	348.68	348.68	14.5**	8.2501	8.2501	10.2**
Nu. vs. S	2	279.17	139.59	5.8*	5.9271	2.9636	3.7
S SO$_2$ Nu.	2	192.68	96.34	4.0*	3.1969	1.5989	2.0
S Nu. H$_2$O	2	17.48	8.74		1.2031	0.6015	
H$_2$O SO$_2$ Nu.	1	7.62	7.62		0.2945	0.2945	
H$_2$O SO$_2$ S	2	3.26	1.63		0.2225	0.1112	
Error	26	627.74	24.14		20.9997	0.8076	
Total	47	10176.66			277.0060		

* Significant

sulphur concentration. The effect of water supply at the different levels of nutrient supply is of great significance.

SULPHUR CONTENT OF PLANTS

The alfalfa used in experimental fumigations 4 and 5 was analyzed for total sulphur by A. N. Johnson of this Institute who used the Parr bomb

TABLE VIII

EFFECT OF SO₂ ON SULPHUR CONTENT OF ALFALFA USED IN EXPERIMENTS 4 AND 5.
PER CENT TOTAL SULPHUR (OVEN DRY BASIS—LEAVES AND STEMS)

			Experiment 4			Experiment 5		
			S+	S	S−	S+	S	S−
Control	Ample water	Full nutrient supply	0.499	0.224	0.259	0.612	0.366	0.268
		Deficient nutrient supply	0.404	0.394	0.335	0.600	0.375	0.433
	Less water	Full nutrient supply	0.452	0.305	0.292	0.728	0.426	0.392
		Deficient nutrient supply	0.457	0.452	0.445	0.575	0.516	0.756
		Average	0.453	0.344	0.333	0.629	0.421	0.462
Treated (SO₂)	Ample water	Full nutrient supply	0.622	0.555	0.462	0.664	0.607	0.593
		Deficient nutrient supply	0.405	0.592	0.599	0.697	0.538	0.598
	Less water	Full nutrient supply	0.607	0.422	0.475	0.852	0.932	0.581
		Deficient nutrient supply	0.577	0.587	0.617	0.737	0.521	0.478
		Average	0.552	0.539	0.538	0.738	0.650	0.563

Note: Sulphur concentrations of the nutrient solutions were 0.083, 0.033, and 0.013 g. per 100 cc. of solution and are designated S+, S, and S−, respectively.

ANALYSIS OF VARIANCE

Source of variation	Degrees of freedom	Experiment 4			Experiment 5		
		Sum of squares	Mean square	F	Sum of squares	Mean square	F
SO₂	1	0.167000	0.167000	18.3**	0.130685	0.130685	9.0*
Sulphur	2	0.022283	0.011142	1.2	0.135902	0.067951	4.7*
Nutrient conc.	1	0.019837	0.019837	2.1	0.001962	0.001962	
H₂O	1	0.004760	0.004760		0.056357	0.056357	3.9
SO₂ vs. S	2	0.013606	0.006803		0.022346	0.011173	
S vs. Nu.	2	0.000287	0.000144		0.048984	0.024492	1.7
H₂O vs. S	2	0.002346	0.001173		0.003746	0.001873	
SO₂ vs. Nu.	1	0.002054	0.002054		0.054435	0.054435	3.7
SO₂ vs. H₂O	1	0.002360	0.002360		0.004134	0.004134	
H₂O vs. Nu.	1	0.009362	0.009362		0.009558	0.009558	
Error	9	0.082240	0.009138		0.130658	0.014516	
Total	23	0.326135			0.598767		

* Significant.
** Highly significant.

method as described by McCool and Johnson (10). In order to obtain large enough samples for analysis, plants treated in duplicate were ground together and used as one sample. Results of the analyses are given in Table VIII.

Examination of the analysis of variance of the sulphur content data for experimental fumigation 4 reveals that the effect of treatment with SO_2 is highly significant, but that the effect of sulphur content of nutrient supply is not significant.

It is believed that the effects of sulphur content of nutrient supply were obscured by the absorption of sulphur from the sand and from the clay pots which were shown to have contained considerable sulphur. (See under "Absorption of Sulphur Dioxide by Sand.")

Examination of the analysis of variance of the sulphur content data for experimental fumigation 5 reveals that both the treatment with sulphur dioxide and the sulphur content of nutrient supply had significant effects on the total sulphur content of the alfalfa.

NITROGEN CONTENT OF PLANTS

Some of the alfalfa used in experimental fumigation 5 was analyzed for nitrogen by A. N. Johnson who used the Kjeldahl method as described by McCool and Johnson (10). Although plants treated in duplicate were ground together and used as one sample for analysis, the samples grown with a deficient supply of nutrients were not large enough to permit analysis, so only figures for plants grown with a full supply of nutrients are available in the data given in Table IX.

Examination of the analysis of variance of the nitrogen content data reveals that neither treatment with sulphur dioxide, nor sulphur content of nutrient supply, nor water supply had any significant effect on nitrogen content of the alfalfa.

The interaction SO_2 vs. S gains significance because the sulphur dioxide increased the nitrogen content of the alfalfa grown with normal supply of sulphur in nutrient solution, decreased slightly the nitrogen content of the alfalfa grown with an excess of sulphur in the nutrient solution, and had no effect on the nitrogen content of the alfalfa grown with a deficient supply of sulphur in the nutrient solution. In the absence of confirmatory data, no great importance can be attached to the significance of this interaction.

ABSORPTION OF SULPHUR DIOXIDE BY SAND

The question arises as to whether the increases in yield of sulphur dioxide treated plants were due to utilization of sulphur obtained directly from the air or whether the plant used only sulphur which had been absorbed through the soil.

A series of 5-inch clay pots containing sand only were subjected to

sulphur dioxide and the sand then analyzed for sulphur by the method of Schroeder (14) under the direction of A. Mehlich of this Institute. After seven days' exposure to 0.10 p.p.m. of SO_2, pots which were saturated with distilled water all during treatment showed an increase in sulphur content over control pots of 3.1 mg. per pot. Pots which received no water showed an increase in sulphur content of 0.7 mg. per pot.

TABLE IX

EFFECT OF SO_2 ON NITROGEN CONTENT OF ALFALFA USED IN EXPERIMENT 5.
PER CENT NITROGEN (OVEN DRY BASIS—LEAVES AND STEMS)

		S+	S	S−
Control	Ample water	4.75	4.00	4.40
	Less water	5.02	3.78	4.79
	Average	4.89	3.89	4.60
Treated (SO_2)	Ample water	4.44	4.68	4.41
	Less water	4.52	4.65	4.61
	Average	4.48	4.67	4.51

Note: Sulphur concentrations of the nutrient solutions were 0.083, 0.033, and 0.013 g. per 100 cc. of solution and are designated S+, S, and S−, respectively.

ANALYSIS OF VARIANCE

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
SO_2	1	0.0271	0.0271	2.3
Sulphur	2	0.3421	0.1711	14.3
H_2O	1	0.0397	0.0397	3.3
H_2O vs. S.	2	0.0938	0.0469	3.9
SO_2 vs. S	2	0.7449	0.3725	31.2*
H_2O vs. SO_2	1	0.0029	0.0029	
Error	2	0.0238	0.0119	
Total	11	1.2743		

* Significant.

With SO_2 treatment of 1.00 p.p.m., the water-saturated pots showed an increase in sulphur content of 14.2 mg. per pot after five days' exposure. Dry pots showed an increase of 5.4 mg. per pot. The sulphur content of unwashed, untreated sand averaged 7.7 mg. per pot.

It is probable that some of the sulphur absorbed by the soil was taken up by the roots of the plants. The fact that plants receiving less water showed the greater growth response to sulphur dioxide treatment, and the fact that sulphur absorption from the soil varies directly with water supply, suggests, however, that the plants utilized some of the sulphur which they absorbed directly from the air as sulphur dioxide.

DISCUSSION OF RESULTS

Under none of the varied experimental conditions studied did treatment with low non-marking concentrations of sulphur dioxide result in a significant decrease in yield of alfalfa, and under certain conditions treatment with the gas brought about significant increases in alfalfa yield.

Although the increase in yield in any one experiment is not significantly different at the various levels of sulphur content of nutrient supply, the consistently greater increase in yield of alfalfa grown with a deficient supply of sulphur is an indication that deficiencies in available sulphur were made up by absorption of sulphur dioxide from the air. The highly significant effect of sulphur dioxide in experiment 2 is an indication that alfalfa growing rapidly under favorable light conditions responds more readily to the growth effect of the SO_2 than does alfalfa growing slowly under less favorable light conditions.

Alfalfa grown with a deficient supply of balanced nutrients did not respond any differently to the yield effect of sulphur dioxide than did alfalfa grown with an ample balanced nutrient supply.

Differences in water supply were not large enough to produce significant changes in yield except in fumigation 5. In this experiment, the alfalfa grown with a deficient supply of water responded to the growth influence of the sulphur dioxide, whereas the alfalfa grown with ample water supply showed no effect.

The increase in yield of the Cruciferae treated with SO_2 in fumigation 3 was not large enough to be significant.

SUMMARY

The effects of prolonged exposure to low non-marking concentrations of sulphur dioxide have been studied, using alfalfa and Cruciferae grown under differing conditions. Water supply, nutrient supply, sulphur content of nutrient supply, and age of plants were systematically varied to enable comparison of the effects of sulphur dioxide under the several conditions and to permit statistical analysis of the yield data.

Under none of the conditions studied did sulphur dioxide treatment decrease the yield of alfalfa, under most of the conditions it had no significant effect on yield, and under some of the conditions it brought about significant yield increases. The data indicate that deficiencies in sulphur content of nutrient supply were made up by absorption of sulphur dioxide from the air. Age of plants and the amount of supplied nutrients had no effect on the yield response to the gas treatment. Alfalfa grown with a deficient supply of water seemed to respond more readily to the stimulating effect of the sulphur dioxide than did plants grown with an ample water supply. Alfalfa growing rapidly under favorable light conditions

responded more readily to the growth effect of SO_2 than did alfalfa growing slowly under less favorable light conditions.

Treatment with sulphur dioxide had no significant effect on yield of Cruciferae.

Analyses of the sulphur content of the alfalfa show that treatment with sulphur dioxide brought about significant increases in per cent total sulphur. The only other variable affecting sulphur content was the sulphur contained in the nutrient supply.

Nitrogen analyses of alfalfa indicate that neither sulphur dioxide, water supply, nor sulphur content of nutrient supply had significant effect on nitrogen content of the alfalfa.

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VIABILITY OF STORED LILIUM POLLEN

Norma E. Pfeiffer

It was previously found (2) that the viability of pollen in four species of *Lilium* and in hybrid *Amaryllis* could be conserved well by storage 1) in desiccators at 10° C. in relative humidities of 35, 50, and 65 per cent; 2) in gelatin capsules at −5° C. or −11° C. with no humidity control; 3) under reduced pressure at −5° C. (for *Lilium*). The test for viability used was ability of the pollen grains to germinate on a hanging drop of an artificial medium consisting of 7 per cent cane sugar, 1.5 per cent gelatin with the addition of a small amount of boiled yeast, for *Lilium*, and of 7 per cent cane sugar, 1 per cent agar, and yeast for *Amaryllis*.

In the desiccators, humidities were controlled by means of concentrated sulphuric acid and definite concentrations of the acid. In the course of the work, it seemed that some other chemical might prove more convenient and practical for the grower who desired to store pollen. Saturated salt solutions were tried and the results obtained upon storage of pollens over these solutions are given in the first section of this paper.

Meanwhile, some lots of *Lilium* pollen originally reported upon gave continued evidence of ability to germinate on an artificial medium after eight and nine months' storage. The results of controlled pollinations made with this stored pollen constitute the second section of this paper.

The work was further extended by storage of pollen according to the simple method suggested by Horsford (1) who saved *L. auratum* pollen over winter by wrapping it in several sheets of paraffin paper. The third section of this paper presents the data on controlled pollinations using pollen stored at different temperatures by this method.

I. GERMINATION AFTER STORAGE OVER SATURATED SALT SOLUTIONS

Since relative humidities of 35, 50, and 65 per cent had been found advantageous in the desiccators made up with concentrations of sulphuric acid, humidities approximating these were used in the new series. A saturated aqueous solution of a given salt with an excess of the solid at a given temperature maintains a constant humidity within any enclosed space about it. The selection of salts to be used was based not only on the humidities desired, but also on the solubility, ready availability, and relative low cost of the salt. Desiccators were made up with saturated solutions with an excess of the chemical for the following salts which give these humidities at the temperatures indicated (3):

Magnesium chloride—35% humidity at 10° C.; 33% at 20° C.
Calcium chloride—38% humidity at 10° C.; 32.3% at 20° C.

Potassium carbonate—44% humidity at 18.5° C.; 43% at 24.5° C.
Potassium thiocyanate—47% humidity at 20° C.
Sodium dichromate—52% humidity at 20° C.
Magnesium nitrate—56% humidity at 18.5° C.
Ammonium nitrate—ca. 68% humidity at 10° C.; 64% at 20° C.

All were used in a dark room automatically controlled at 10° C., with a comparison set of desiccators made up with concentrations of sulphuric acid to give humidities of 35, 50, and 65 per cent. In addition a set of desiccators in darkness at room temperature included the same concentrations of sulphuric acid and saturated solutions of potassium carbonate and sodium dichromate.

For convenience, desiccators where the humidity was controlled by salts will be referred to by the name of the salt, and those controlled by concentrations of sulphuric acid by the humidities obtained.

Pollen from the following forms was used: *Lilium longiflorum* Thunb., *L. auratum* Lindl., *L. philippinense* Baker, and *L. speciosum* Thunb., var. *rubrum* Hort. *L. longiflorum* pollen was stored in May, *L. auratum* in July, and that of the other two forms in September, 1936. Pollen of hybrid *Amaryllis* was stored in spring of the same year.

The test for viability as in the earlier work was ability of the pollen grains to germinate on hanging drops of the cane sugar, gelatin or agar, yeast medium. Tests were run in duplicate and counts made of the grains which germinated as compared with the total number in a representative number of fields.

L. longiflorum. Since pollen of this form was early available in quantity, it was stored in all desiccators of the series and in gelatin capsules in an electric refrigerator, recording about 7° C., and tested at intervals over a period of 14 months. There was a gradual decrease in the percentage of pollen grains germinating as the storage interval increased. Successive tests gave fairly consistent results.

At the end of 11 months, the following storage conditions gave very good germination, approximating 50 per cent: gelatin capsule in the refrigerator (7° C.), and at 10° C., magnesium chloride, calcium chloride, potassium carbonate, 35 per cent and 50 per cent humidity. Pollen from the sodium dichromate desiccator (10° C.) showed about 35 per cent germination. Pollen over magnesium nitrate and over sulphuric acid giving 65 per cent humidity, both at 10° C., showed lower germinating power, about 24 per cent germination.

After 14 months, the lots of pollen stored at 10° C. in desiccators having humidities of 35 to 50 per cent, whether controlled by magnesium chloride, calcium chloride, potassium carbonate, or concentrations of sulphuric acid, continued to exceed 45 per cent germination in almost all

tests. At the same time, germination of pollen in the sodium dichromate desiccator dropped to 19 per cent, magnesium nitrate to 9 per cent, and in 65 per cent humidity to 1 per cent. Pollen stored over potassium thiocyanate and ammonium nitrate solutions gave poor results early in the experiment and were no longer tested at this time.

Pollen of this series stored at room temperature for two months gave the best results in the lower range of humidities, 25, 35, 50 per cent, and over potassium carbonate with germination percentages ranging from 8 to 29 per cent. In four months, the germinating ability was much reduced and after seven months, was only about 1 per cent in the best tests.

A comparison with the results of tests in a previous year at 10° C. show a higher percentage of germination in 35 per cent humidity than then reported, and a lower percentage in 65 per cent humidity.

L. auratum. Pollen of this species stored in the same set of desiccators on July 27, gave consistently lower germination percentages than those obtained the previous year. The greatest production of pollen tubes was, however, in the same general range of humidities.

At the end of two months, pollen from the capsule in the refrigerator (7° C.), from 10° C. desiccators with salt solutions, and 35, 50, and 65 per cent humidity, gave good percentages of germination, ranging as high as 64 per cent. After six months' storage the most satisfactory conditions as indicated by germination percentages of 36 and 43 per cent and 30 and 35 per cent respectively were the gelatin capsule in the refrigerator and the 50 per cent humidity at 10° C. Next in order with germination percentages between 20 and 30, were the 10° desiccators with magnesium chloride, calcium chloride, and potassium carbonate. Less than 20 per cent germination resulted with pollen from the other desiccators. After eight months, pollen from the capsule in the refrigerator and from 10° C. desiccators with calcium chloride and with potassium carbonate, still exceeded 25 per cent germination. Pollen over magnesium nitrate and in 35, 50, and 65 per cent humidity, showed 10 to 20 per cent germination. The least satisfactory results were obtained with magnesium chloride and sodium dichromate. Usually magnesium chloride is among the most satisfactory salts, both for *auratum* pollen after shorter intervals of storage and for other species of pollen.

Pollen of *L. auratum* germinated poorly after storage at room temperature for two months with 10 per cent or less of the grains germinating under practically all storage conditions. Results were negative in all germination tests at four months.

L. speciosum var. rubrum. Pollen of this form was tested after seven and ten months' storage. Lower percentages of germination with more variation resulted than in tests the previous year. Most satisfactory results were obtained at seven months with pollen stored in a gelatin cap-

sule in a refrigerator (7° C.), at 10° C. over magnesium chloride, calcium chloride, potassium carbonate and at 50 per cent humidity. After ten months, magnesium chloride and calcium chloride appeared slightly better than potassium carbonate, 50 per cent and 35 per cent humidity. Pollen in other storage conditions, including the gelatin capsule in the refrigerator, had dropped to 1 per cent germination or less.

L. philippinense. Stored pollen of this species showed greater ability to germinate than that stored the previous year. After nine months, the best germination was obtained over the following, at 10° C.: magnesium chloride, 65 and 74 per cent; calcium chloride, 66 and 74 per cent; potassium carbonate, 51 and 59 per cent. Next in order were magnesium nitrate, 46 and 52 per cent; 50 per cent humidity, 36 and 55 per cent; sodium dichromate, 37 and 46 per cent; and 35 per cent humidity, 22 and 51 per cent. These average from 70 per cent in the most successful to 35 per cent in the least. Lower percentages were obtained in 25 per cent humidity controlled by sulphuric acid, 16 and 22 per cent, and 65 per cent humidity with 9 and 11 per cent. There was no storage of this pollen at refrigerator temperature or at room temperature.

Hybrid Amaryllis. As in the earlier work, a limited amount of pollen of hybrid *Amaryllis* was stored. No single collection sufficed for samples for all the desiccators. In one lot, good germination resulted after more than seven months' storage at 10° C. in 35 per cent humidity, 63 and 64 per cent; potassium carbonate, 47 and 54 per cent; and at − 5° C. without humidity control, 52 and 59 per cent. This group gave somewhat less satisfactory results for the same lot of pollen: gelatin capsule at − 10° C., 32 and 37 per cent; desiccators at 10° C., magnesium nitrate, 30 and 39 per cent; potassium thiocyanate, 28 and 32 per cent; 50 per cent humidity, 24 and 31 per cent; sodium dichromate, 17 and 22 per cent. Least satisfactory were 25 per cent humidity, 15 and 16 per cent, and 65 per cent humidity, 1 and 5 per cent.

Another series at 10° C. gave results in this order: potassium carbonate, 69 and 70 per cent; potassium thiocyanate, 57 and 59 per cent; magnesium nitrate, 26 and 33 per cent; and sodium dichromate, 16 and 17 per cent.

A third lot of pollen stored more than seven months at 10° C. over magnesium chloride showed 42 and 43 per cent germination; calcium chloride, 39 and 41 per cent; in 50 per cent humidity, 42 and 47 per cent. Still another showed 33 and 34 per cent germination after storage over magnesium chloride, 42 and 50 per cent over calcium chloride, after almost five months' storage.

The results of germination of *Amaryllis* pollen on an artificial medium are in accord with the field results of Traub (4) who reports best results in pollinations made after storage at 10° C. over saturated solutions of calcium chloride and magnesium chloride. For comparison he used only solu-

tions (chlorides of zinc and lithium) providing lower humidities of about 10 and 15 per cent, which in our previous experience with humidities controlled by sulphuric acid, were too low to be effective over long intervals.

It is concluded from the data obtained with pollen of the forms used that saturated solutions of magnesium chloride, calcium chloride, and potassium carbonate, are suitable for humidity control at 10° C., giving as good results as the most favorable concentrations of sulphuric acid. Solutions of magnesium nitrate and sodium dichromate are next in rank. For *Amaryllis* pollen, potassium thiocyanate can be classed with these. But this solution is considered unsuitable for long storage of *Lilium* pollen because of sharp declines in germinating power after six and seven months. Ammonium nitrate also proved unsatisfactory, early in the course of the work, doubtless due to its unstable nature.

It must be pointed out that good results are sometimes obtained with storage of *Lilium* pollen in a gelatin capsule at 7° C., with no humidity control. The oily character of this pollen tends to hold the grains in a close mass and to protect them somewhat against loss of water, especially when storage is not too prolonged.

II. POLLINATIONS WITH POLLEN STORED IN CONTROLLED HUMIDITIES AND IN VACUUM

Good germination of pollen on the sugar-gelatin-yeast medium gave continued evidence of the ability of the grains to produce apparently functional tubes after storage for eight or nine months in some of the conditions of the original experiment. This method is a convenient one, available for testing viability at any time of year. For comparison, controlled pollinations were made with the stored pollen on hand, as flowers of the same species came into bloom.

L. longiflorum. In the greenhouse from the middle of April into May, all flowers were emasculated in bud and pollinations were made with pollen stored the previous year at humidities of 50 and 65 per cent at 10° C. and under reduced pressure at 5° C. and 20° C. In most cases the pollen was applied on the moist stigma with no deviation from the usual procedure. In a few cases, the style was cut, a portion removed, the upper part replaced and held in position by a dandelion scape; the pollen was applied to the stigma of the much shortened style. In three of seven trials, a small amount of seed was set, showing that the connection was close enough to allow the tubes to function. This procedure was not repeated since it was found that the pollen tubes grew adequately in length and rate for the normal length of the style.

Eight flowers pollinated with pollen stored since the preceding August in a vacuum at 5° C. produced eight capsules with 764 seeds. With pollen stored at 10° C. in 65 per cent humidity, 12 capsules resulted from 21 pollinations, producing 435 seeds. Pollen stored at 10° C. in 50 per cent

humidity was available in only very small amount, and was probably spread too much in attempting ten pollinations, of which only three took, with 28 seeds produced. There was no capsule or seed production when pollen from 20° C. in a vacuum was used. Representative capsules are shown in Figure 1.

Longiflorum seed produced by means of pollen stored at 65 per cent humidity, 10° C., showed 75 per cent germination; from 5° C. vacuum, showed 74 per cent; and from 50 per cent humidity, 43 per cent germination.

L. auratum. Flowers of this species were available in quantity on August 20. Emasculated and bagged flowers were pollinated with pollen stored twelve months earlier at 10° C. in 35 and 50 per cent humidity, with two lots stored in gelatin capsules with no humidity control at −11° C. (first two months either at −5° C. or −17° C., depending upon the lot), and with pollen under reduced pressure at 5° C. and 20° C. Since no tube still sealed under reduced pressure at −5° C. was available, one which had been opened for sampling the latter part of March and then plugged with cotton, was used to represent this condition. Five pollinations were made in each case except those pollens in 35 and 50 per cent humidity where barely enough was available for three stigmas each.

Five capsules resulted from five tries with pollen from reduced pressure at 5° C. (1636 seeds) and at −5° C. (1479 seeds) and from normal pressure in gelatin capsules at −11° C. with first two months at −5° C. (1054 seeds). Pollen from gelatin capsules originally at −17° C. for two months followed by ten months at −11° C. was less effective as indicated by four takes out of five (458 seeds), smaller average size of capsules, and more chaff. Capsules produced by the use of pollen stored under these conditions (reduced pressure and gelatin capsules) are shown in Figure 2 A and B. Germination of seeds produced by these pollens ranged from 72 to 76 per cent showing fairly uniform results in the four cases.

When pollen from 35 and 50 per cent humidity was used, no seeds resulted although pollination stimulated growth of two chaffy capsules out of three trials in each case; those produced as the result of pollen stored in 50 per cent humidity are shown in Figure 2 A. Neither capsule nor seeds resulted with the use of pollen stored at 20° C. in a vacuum.

L. speciosum var. rubrum. From August 12 to 20, 1936, similar pollinations were made on flowers of this variety with pollen of the same sort stored in August 1935, under reduced pressure at 5° C. and −5° C., under atmospheric pressure in gelatin capsule at −11° C. (originally −17° C.) and in controlled humidities of 35 and 65 per cent at 10° C.

Nine takes (1415 seeds) from fifteen tries with pollen from −5° C. in vacuum, five takes (708 seeds) from ten tries with pollen from 5° C. in vacuum, and five takes (252 seeds) from five tries with pollen in a gelatin
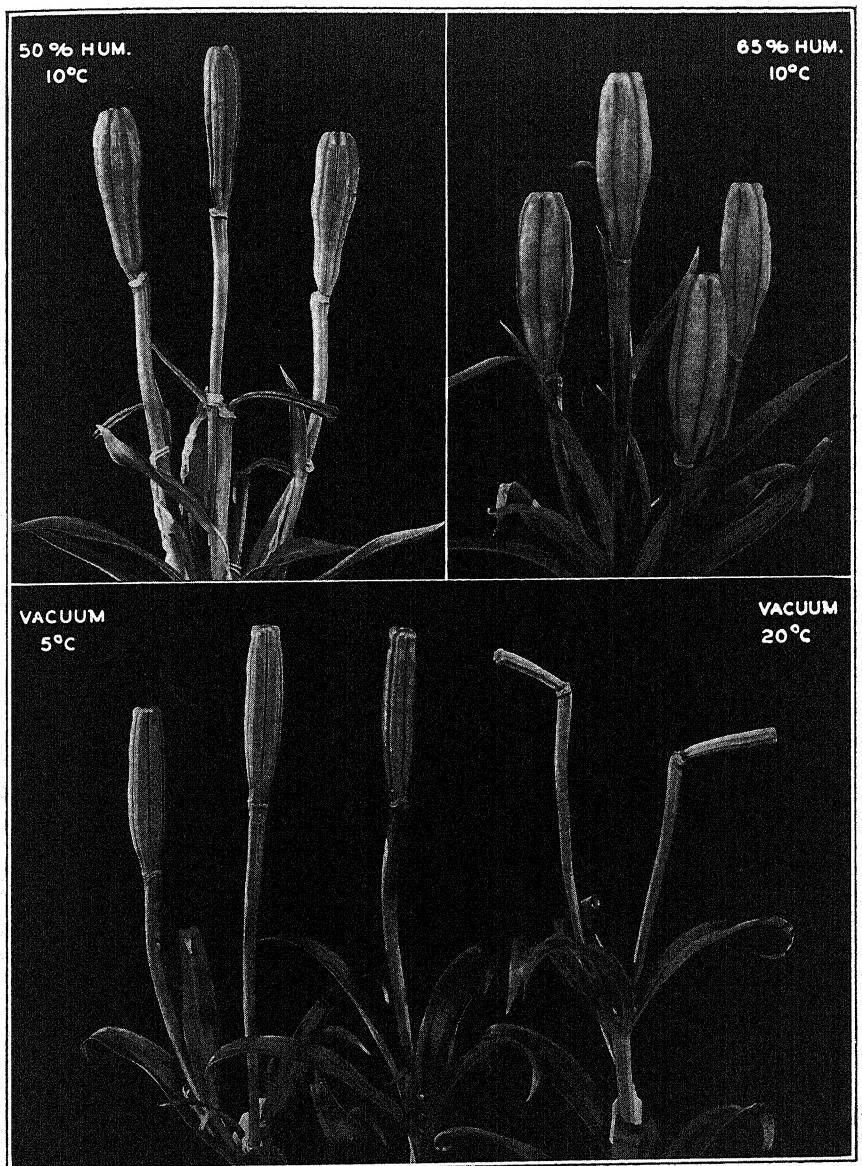
FIGURE 1. *L. longiflorum* capsules produced by means of pollen stored the previous August; pollinations April 25 to May 19. Photographed June 19.
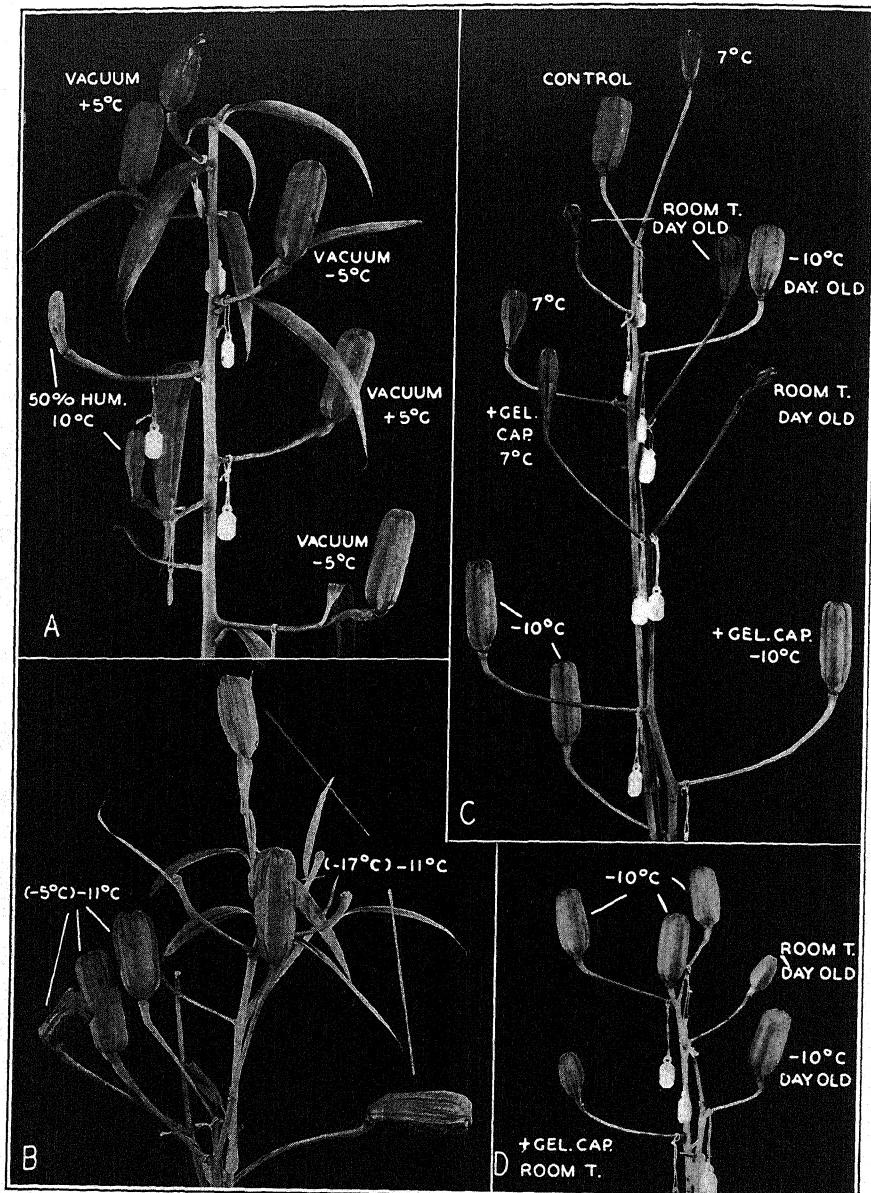
FIGURE 2. *L. auratum* capsules resulting from pollinations made July 30 to August 20 with pollen stored the previous year. A. Storage in August, under the conditions indicated in the labels; B. Storage in August, in gelatin capsules; C and D. Storage in September in paraffin paper only, except where marked plus gelatin capsule; control capsule produced with fresh pollen of the current year.
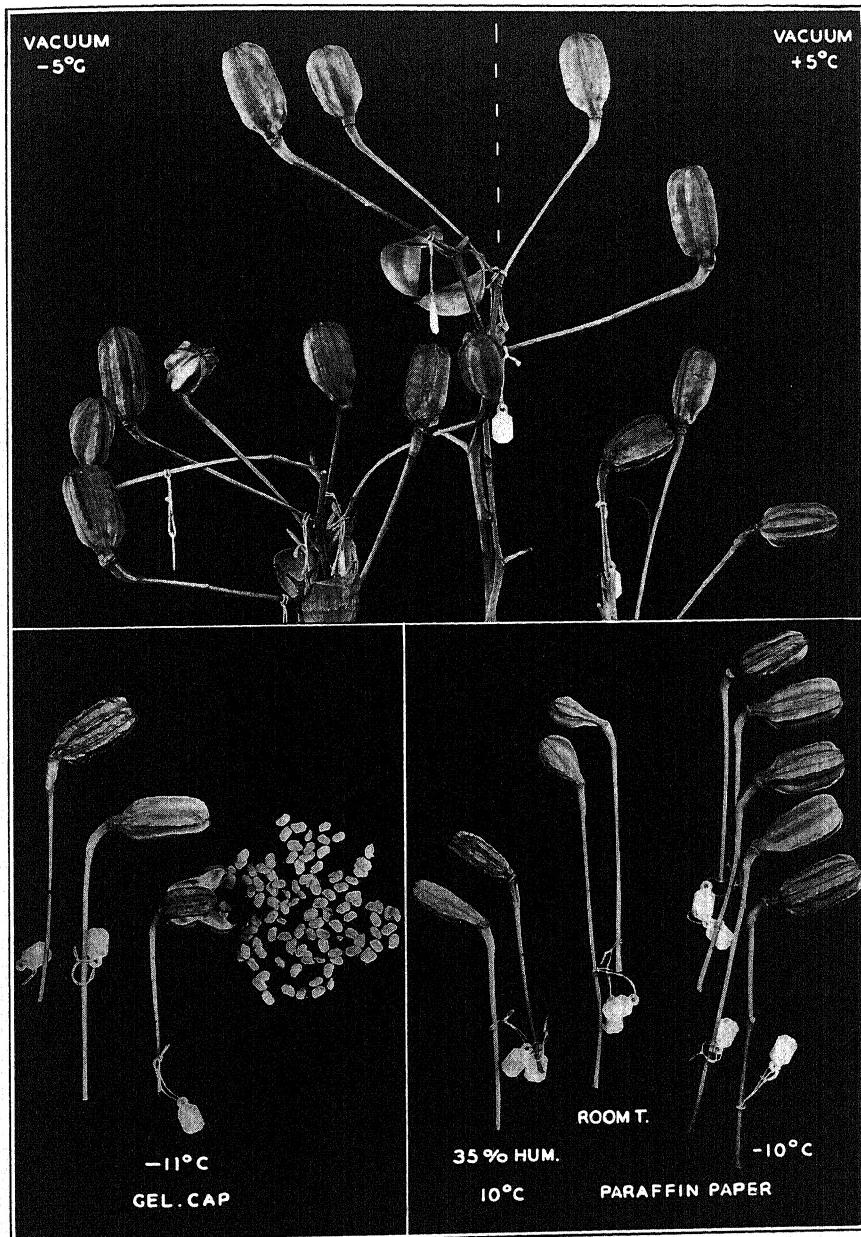
FIGURE 3. *L. speciosum* var. *rubrum* capsules produced by means of pollen stored the previous August. Pollinations in August.

capsule at $-11°$ C., show the best of the conditions used for preserving viability of the pollen (Fig. 3). Five and three flowers respectively were pollinated with pollen from 35 and 65 per cent at 10° C., because of small amounts of pollen available. Two capsules with 22 seeds resulted in the case of pollen from 35 per cent humidity (Fig. 3).

Germination percentages were 79 per cent for seeds produced by use of pollen from $-5°$ C. in vacuum, 65 per cent from 5° C. in vacuum, and 86 per cent from gelatin capsule at $-11°$ C.

L. philippinense. Flowers in bloom in a gauze-covered cage during late August were emasculated early but not bagged. Pollen was available from humidities of 35, 50, and 65 per cent at 10° C.; from vacuum at 5° C. and also from $-5°$ C. sealed until the end of March and then plugged with cotton. Five pollinations were made in each case except for four at 35 per cent humidity and three at 50 per cent humidity, with the following results: vacuum $-5°$ C., five capsules, 4805 seeds; vacuum 5° C., four capsules, 3889 seeds; 35 per cent humidity, two capsules, 192 seeds; 50 per cent humidity, three capsules, 94 seeds; and 65 per cent humidity, four capsules, 893 seeds. Unquestionably, the pollen stored under reduced pressure gave the largest seed production.

Germination tests of seeds from each group gave 94 to 99 per cent germination with the exception of seeds resulting from pollen stored at 50 per cent humidity, with 68 per cent germination.

DISCUSSION

No accurate comparison can be made between the percentage of pollen grains germinating under natural conditions upon the stigma and that on an artificial medium. However, the set of seed in some cases was greater than would have been anticipated from the last germination percentages obtained on the sugar-gelatin-yeast medium. This was notable in the cases of storage of pollen with no humidity control under reduced pressure at 5° C. or $-5°$ C. and in gelatin capsules with atmospheric pressure at temperatures below freezing ($-11°$, $-5°$ C.). The pollen stored in controlled humidities was available in lesser amounts and had also undergone slight exposure to other conditions of temperature and moisture at the times of sampling for germination during the preceding twelve months. It is possible that the small number of pollen grains was responsible for the small number of seeds produced after pollen storage at 10° C. in controlled humidities.

Advantage has been taken of some of these methods in the storage of pollen of other species of *Lilium* for use in interspecific pollinations. Positive results have been obtained with pollen stored 71 days at 10° C. in 50 per cent humidity, others stored up to 289 days at 10° C. in 65 per cent humidity, and up to 301, 352, and 365 days at $-10°$ C. in gelatin capsules.

It must be noted that these data do not indicate relative effectiveness, since the chance of setting seed in these cases involves other factors of compatibility and sterility. Positive results, however, indicate longevity of pollen under the particular conditions used.

III. CONTROLLED POLLINATIONS WITH POLLEN STORED IN PARAFFIN PAPER

Controlled pollinations were also used as a means of determining the effectiveness of a method of pollen storage reported by Horsford (1). He found it possible to save *L. auratum* pollen from late one year until the following spring for use on early blooming lilies by means of wrapping it in several sheets of paraffin paper. Pollen of *L. auratum* and *L. speciosum* var. *rubrum* were used.

L. auratum. On September 11, freshly collected stamens of *L. auratum* were allowed to stand till the pollen was fairly dry, then divided into lots, three of which were placed directly in several wrappings of paraffin paper. Paper packets were placed directly in stoppered vials for storage at the following temperatures: 1) room temperature; 2) refrigerator, 7° C.; 3) −10° C. Pollen was also stored at −10° C. enclosed in a gelatin capsule which was then wrapped in paraffin paper. Stamens and pollen of the same collection allowed to dry at room temperature till the following day were similarly wrapped in paraffin paper only, and stored at room temperature and at −10° C.

Tests of germinating ability made on the sugar-gelatin-yeast medium the following June gave negative results with pollen stored at room temperature, 6 and 7 per cent in the refrigerator, 32 and 38 per cent in paper at −10° C. with pollen fresh at the time of storage, 20 and 34 per cent with pollen a day old at storage, 28 and 28 per cent at −10° C. for pollen within capsule and paper.

At the end of July and beginning of August when the pollen had been stored more than ten months, pollinations were made using flowers which were emasculated and bagged prior to and following application of stored pollen. At this time, the pollen from room temperature and refrigerator appeared dry and discolored, that in −10° C. retained the original bright color. There was some capsule and seed production from pollen of each storage condition, but with great difference in size of pod and proportion of seed and chaff. The most successful were those resulting after storage at −10° C. whether fresh or day-old pollen was stored (Fig. 2 C and D). Ten tries with pollen in gelatin capsule plus paraffin paper gave ten pods with about 3200 seeds, twenty tries with pollen in paper yielded twenty pods with about 3400 seeds, while thirteen tries with day-old pollen in paper gave thirteen pods with about 1950 seeds. On the other hand, with pollen from the refrigerator (7° C.), thirteen pollinations resulted in but

eight pods with 65 seeds. Pollen at room temperature gave less satisfactory results, producing two pods with 21 seeds when stored fresh and five pods with 52 seeds when a day old at storage.

Germination of the seed produced was almost 75 per cent for seed resulting from pollen stored fresh at $-10°$ C. in paper only, 47 per cent in that stored after drying in air 24 hours, and 33 per cent in paper plus gelatin capsule. With the small numbers of seed available from pollen stored at room temperature and in refrigerator, 62 and 54 per cent germination were obtained respectively.

If comparison is made of the number of *auratum* seedlings obtained per pollination (in a single germination test in each case) through the use of pollen from all the various storage conditions at temperatures lower than room temperature, tried in Sections II and III, the order from greatest to least runs thus: (1) with pollen under reduced pressure either at $5°$ C. or $-5°$ C.; (2) with atmospheric pressure at $-5°$ to $-11°$ C. in gelatin capsule; (3) at $-10°$ C., either in paraffin paper or gelatin capsule within paraffin paper; (4) at $-10°$ C. in gelatin capsule alone and day-old pollen at $-10°$ C. in paraffin paper; (5) and least at refrigerator temperature ($7°$ C.) in paraffin paper.

L. speciosum var. rubrum. Pollen was stored only at room temperature and at $-10°$ C., in each case wrapped in several layers of paraffin paper either with or without a gelatin capsule; it was stored soon after being collected in September. Tests for germination made the following June on the cane sugar-gelatin-yeast medium gave negative results for that stored at room temperature and 5 and 10 per cent in duplicate tests for pollen at $-10°$ C.

Controlled pollinations were made the following August. Two series of tests in different locations with pollen from $-10°$ C., within a gelatin capsule wrapped in paraffin paper, gave nine pods (about 1250 seeds) from nine tries and five pods (about 775 seeds) from five tries (Fig. 3). Pollen from $-10°$ C. within paraffin paper only gave six somewhat chaffy pods (203 seeds) from seven tries. Pollen from room temperature in ten trials produced two small pods with 22 seeds, all viable. Germination tests involving larger numbers of seeds resulting from pollen stored at $-10°$ C. gave 90 per cent germination in each case.

The results show that the combined use of gelatin capsules and paraffin paper for storage of *Lilium* pollen at $-10°$ C. is more favorable than paper alone or capsule alone. In either case, loss of moisture may be involved, since there is some evidence of adherence of pollen to the lightly paraffined paper, and the capsules in themselves are not proof against water loss. Results from storage at $-10°$ C. are much more successful than storage within paraffin paper in a refrigerator at $7°$ C. or at room temperature. Furthermore, drying of the pollen for 24 hours after collecting reduces the amount of good seed per pollination.

GENERAL SUMMARY

Pollen of *Lilium* species stored for the greater part of a year remained viable, and when applied to stigmas yielded seed which gave a high percentage of germination and produced strong seedlings.

Controlled pollinations made with such pollen lead to these conclusions:

1. Storage of pollen under reduced pressure at 5° or −5° C. can be especially recommended for long intervals. Capsules, with a good set of seeds, were formed in every case. On the other hand, storage in vacuum at 20° C. gave negative results for *L. auratum* and *L. longiflorum*.

2. Pollen can be stored with less expenditure of labor at −10° C. or −11° C., especially when the pollen is enclosed in a gelatin capsule, which is then wrapped in paraffin paper. This is more satisfactory than pollen either within gelatin capsule alone or paraffin paper alone, for the two species tried.

3. Pollen of *L. auratum*, wrapped in paraffin paper, at refrigerator temperature (7° C.), produced little seed, that at room temperature even less.

4. Storage of pollen at 10° C. in humidities of 35, 50, and 65 per cent can be recommended as a third method. Variable results obtained for the four species, as well as the small amount of seed set, may be related to the small amount of pollen available. This method used with larger amounts of pollen of other species has given set of seed after intervals up to 289 days of storage.

The humidity in these cases was controlled by means of concentrations of sulphuric acid. Saturated solutions of magnesium chloride, calcium chloride, and potassium carbonate were found to give as good results as sulphuric acid concentrations when viability was determined by means of germination of pollen on hanging drops of a solution of 7 per cent cane sugar, 1.5 per cent gelatin plus boiled yeast. The saturated solutions are easier to prepare and to maintain and are recommended as a means for controlling humidity in desiccators for storage of pollen of the four species of *Lilium* used and of hybrid *Amaryllis*. Magnesium nitrate and sodium dichromate solutions preserve viability in lesser degree (10 to 15 per cent lower germination in general); potassium thiocyanate ranks with these for storage of *Amaryllis* pollen.

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EVIDENCE THAT PLANT TISSUE FORMS A CHLORINE-CONTAINING β-GLUCOSIDE FROM ETHYLENE CHLORHYDRIN[1]

LAWRENCE P. MILLER

Considerable quantities of ethylene chlorhydrin are absorbed by potato tubers (*Solanum tuberosum* L.) when they are exposed to the chlorhydrin under conditions which have been found to break the rest period (2, 6, 13). Since chlorhydrin forms a constant boiling mixture of minimum boiling point with water, the absorbed chlorhydrin can readily be recovered from the treated tubers by distillation. Determinations of the amount of chlorhydrin present at intervals after the end of treatment have shown (11) that the chlorhydrin content decreases rapidly after treatment so that after about a week or ten days no unaltered chlorhydrin remains in the treated tubers. It has been found that only a small amount of the absorbed chlorhydrin is subsequently given off by the tubers in the form of vapor into the surrounding air and that the greater part of the chlorine from the absorbed chlorhydrin is left in the tubers.

Analyses of treated and control tubers for total chloride (after unaltered chlorhydrin is no longer present) by the method of Sunderman and Williams (16), which includes a preliminary heating with normal KOH before the use of an open Carius method, have shown the presence of the additional chlorine from the absorbed chlorhydrin. A chlorine determination by the method of Sunderman and Williams includes not only inorganic chloride but also any chlorine present in organic combination of such a nature that heating with KOH converts it into an ionic form. Total chloride determinations by an open Carius method with and without previous treatment with KOH on control tissue and on treated tissue after unaltered chlorhydrin was no longer present have shown (12) that the chlorine from the chlorhydrin remains in a non-ionic form and that control tissue contains no non-ionic chlorine.

Unpublished results have shown that the corms of *Gladiolus*, the rest period of which can also be broken by ethylene chlorhydrin (3, 5), can absorb much larger quantities of the chlorhydrin without injury than potato tubers and that the chlorhydrin content of treated corms decreases more rapidly after treatment than with potato tubers. As with the potato the chlorine remains in a non-ionic form after no unaltered chlorhydrin is left in the gladiolus tissue. Thus, for a study of the compound formed from the chlorhydrin the gladiolus is more favorable material than the potato since larger quantities of the compound can be obtained in a given amount

of tissue. The maximum concentration of chlorhydrin that can conveniently be obtained in the potato without injury is about 160 mg. per 100 g. tissue, while in some of the experiments with gladiolus a concentration of 700 mg. per 100 g. of tissue brought about no injury. The investigations completed at the present time indicate that the compound formed from the chlorhydrin is similar if not identical in the two tissues studied.

The experiments reported in the present paper show that the chlorine-containing compound formed from the chlorhydrin is hydrolyzed by emulsin with the release of a volatile chlorine compound and reducing sugar or at least a substance which reduces Fehling's solution. Preliminary studies on the effect on optical rotation have also shown that on hydrolysis by emulsin there is a shift in optical rotation toward the right. The formation of reducing sugar by emulsin hydrolysis together with a change in optical rotation toward the right is the basis of the method used by Bourquelot [quoted by Klein (9, p. 810)] and his students for demonstrating the presence of glucosides in plants and the results suggest strongly that the chlorine compound in question is a β-glucoside.

METHODS

Gladiolus corms and potato tubers were treated with ethylene chlorhydrin by the methods previously described (2, 6, 13). Subsequent to the end of treatment the material was stored in open containers or in desiccators through which a stream of air was drawn. After unaltered chlorhydrin was no longer present the tissue was either extracted with 80 per cent alcohol or the juice expressed by squeezing through cheesecloth after grinding through a food chopper. To obtain a more complete yield of the compound several portions of distilled water were added to the residue and further extracts obtained.

For the tests with emulsin the hydrolysis was allowed to proceed in a volume of 200 cc. at 31° to 32° C. in the presence of 50 mg. emulsin and about 0.25 millimols of the organic chlorine compound per 100 cc. The solutions were buffered with M/20 acetate buffer at pH 4.75. At the end of the test period an aliquot was taken for the determination of reducing sugar by the method of Quisumbing and Thomas (14) with subsequent determination of the cuprous oxide formed by titration with N/20 KMNO$_4$ after solution in ferric sulphate and sulphuric acid. Another aliquot was taken and distilled to one-half its volume and the distillate heated with an equal volume of N KOH. After cooling, the chloride present was determined by titration by the Volhard method. Controls were carried through the same procedure except that no emulsin was added. In some cases the solutions were also examined with the polariscope to determine the shift in optical rotation produced by the emulsin hydrolysis. For these tests smaller volumes containing higher concentrations of the

organic chlorine compound were employed. Commercial preparations of emulsin were used.

RESULTS

Hydrolysis by sulphuric acid. In the method used for the routine determinations of the amount of chlorhydrin present in tissues or expressed juices of treated material the distillations are carried out at a pH value of about 4.5. As stated in the introduction, under these conditions no volatile chlorine compound is obtained after the chlorhydrin has been altered in the tissue. Experiments have shown, however, that on the addition of appreciable quantities of sulphuric acid a volatile chlorine compound is again obtained on distillation. The data given in Table I were obtained by adding various quantities of sulphuric acid to a concentrated potato extract containing altered chlorhydrin and distilling to half the original volume. The distillate was heated with N KOH and the chloride

TABLE I

HYDROLYSIS BY HEATING WITH SULPHURIC ACID OF CHLORINE COMPOUND FORMED BY POTATO TUBERS FROM ABSORBED ETHYLENE CHLORHYDRIN

H_2SO_4, g. per 100 cc.	Volatile Cl compd. obtained, %
0	Trace
1	Trace
2	1.1
4	8.9
8	58.5
16	83.0
Vol. compound	98.5

determined and percentage recovery calculated on the basis of the non-ionic chlorine known to be present from determinations of the chloride content by an open Carius method with and without previous heating with KOH. The potato extract used was obtained by concentration of expressed juice by evaporation and thus contained many other substances in addition to the non-ionic chlorine compound. The data furnish, however, preliminary information on the ease of hydrolysis of this chlorine compound and show that if over four grams of H_2SO_4 were present in 100 cc. of concentrate over 50 per cent of the non-ionic chlorine was recovered in the distillate. That the low recovery of the volatile chlorine compound with the addition of small amounts of sulphuric acid was not merely due to low volatility of the compound is shown by the last determination given in the table in which some of the volatile chlorine compound previously obtained by distillation with acid was redistilled without the addition of any acid. This shows that the compound once formed is quite volatile and that the low recovery in the presence of small amounts of acid is due to incomplete hydrolysis under these conditions.

The non-ionic chlorine compound formed in gladiolus tissue from ab-sorbed chlorhydrin was similarly hydrolyzed by boiling with sulphuric acid with the recovery of a volatile non-ionic chlorine compound. The identity of the volatile compound thus obtained has not been definitely established but preliminary tests indicate that it may be ethylene chlor-hydrin.

These results, therefore, suggest that the reaction undergone by the absorbed ethylene chlorhydrin in both potato and gladiolus tissue involves the −OH group. Since many substances undergo detoxication in the animal organism through combination with glucose to form the well known glucuronic acid derivatives and since plants can form β-glucosides it was decided to test the action of emulsin on the compound formed in the tissue from the chlorhydrin.

Hydrolysis by emulsin. Because the compound investigated contains chlorine in organic combination and on hydrolysis a volatile chlorine compound is released as well as a product or products which reduce Fehling's solution, it is possible to follow the course of the action of emul-sin by estimation of both the increase in reducing substances and deter-mination of the amount of the volatile chlorine compound formed. Table II shows some of the data obtained by the use of emulsin on extracts ob-tained from gladiolus tissue which contained the product resulting from the absorbed chlorhydrin. The conditions employed for the enzymatic hydrolysis were those described under "methods" except in the first case given in which only 20 mg. of emulsin per 100 cc. were used and in the last experiment shown in which 100 mg. emulsin per 100 cc. were em-ployed. The last three sets of data were obtained from corms of the Sou-venir variety and all the others from the Alice Tiplady variety. Most of the determinations were made with the use of expressed juice which had been heated to boiling to remove substances which precipitate out on heating and which had been subsequently further purified by precipita-tion with lead acetate and removal of excess lead with sodium oxalate. Concentration by evaporation was employed in some cases. Some tests were made on expressed juice directly and others on a butyl alcohol ex-tract (after evaporation of the butyl alcohol) of the expressed juice which had been previously treated with lead as indicated above.

The quantities of the products obtained by the hydrolysis are ex-pressed in the table as millimols. The reducing substances formed were calculated as glucose. Column 4, Table II, lists the millimols of the volatile chlorine compound obtained and the figures in column 8 show the cor-responding quantity of reducing sugar expressed as glucose. The formation of the volatile chlorine compound definitely shows that the compound formed from the ethylene chlorhydrin is attacked by the emulsin but since the enzymatic hydrolyses were carried out on crude preparations the re-

ducing sugar formed does not necessarily all arise from the chlorine compound. It will be noted that the amounts of reducing substances found, calculated as glucose, correspond to considerably more than one molecule of glucose for one molecule of the volatile organic chlorine compound. An understanding of the appearance of the larger quantity of reducing sugar, calculated as glucose, must await investigation with purer, preferably crystalline, preparations of the chlorine compound hydrolyzed by the emulsin. The identity of the sugar formed can then be definitely established. A study of the action of emulsin on extracts obtained from gladiolus corms which had not been exposed to ethylene chlorhydrin showed that emulsin brought about only a slight increase in reducing sugar. Thus the

TABLE II

HYDROLYSIS BY EMULSIN OF ORGANIC CHLORINE COMPOUND FORMED BY GLADIOLUS TISSUE FROM ABSORBED ETHYLENE CHLORHYDRIN

Time, hours	Volatile Cl compd. recovered, millimols			% of org. chlorine recovered	Reducing sugar, as glucose, millimols		
	With emulsin	Without emulsin	Diff.		With emulsin	Without emulsin	Diff.
12.5	0.120	0.005	0.115	22.9	1.11	0.94	0.17
18.0	0.28	None	0.28	53.3	1.45	0.97	0.48
47.0	0.39	Trace	0.39	74.2	1.71	0.99	0.72
17.8	0.34	Trace	0.34	65.4	1.55	0.94	0.61
45.8	0.39	Trace	0.39	74.8	1.73	0.95	0.78
47.0	0.33	Trace	0.33	71.6	0.94	0.38	0.56
17.3	0.38	Trace	0.38	100.0	0.66	0.05	0.61
38.0	0.62	0.09	0.53	31.6	1.64	0.71	0.93
17.8	1.75	Trace	1.75	31.2	4.80	2.02	2.78

action of emulsin on an extract of control tissue corresponding to the extract from the treated tissue shown in line 7 of Table II increased reducing sugars from 0.92 millimol to 1.00 millimol, which corresponds to an increase of only 0.08 millimol and is of course insufficient to explain the extra glucose obtained by emulsin in the treated extract. It will be noted that the sugar content of the control extract is much higher than that of the treated, which agrees with earlier investigations (4) showing that the treated tissue is lower in reducing sugar. Tests with extract of control Alice Tiplady corms also showed only a small increase in reducing sugar from the addition of emulsin. With neither variety is any volatile chlorine compound obtained by the action of emulsin on control material.

Yeast invertase (15) did not hydrolyze the chlorine compound and hydrolysis of the sucrose in the extracts by invertase did not appreciably alter the amount of reducing sugar formed by coincident emulsin hydrolysis.

With the potato lesser quantities of ethylene chlorhydrin can be introduced into the tissue than with gladiolus corms and thus extracts obtained from treated tissue contain a considerably lower concentration of the chlorine compound than with gladiolus. Expressed juice concentrated by evaporation under vacuum, to produce a suitable concentration of the chlorine compound for emulsin tests, thus contains extremely large quantities of impurities including high amounts of reducing sugars. Such preparations were only affected to a small degree by added emulsin. After partial purification, however, emulsin had similar action to that obtained with gladiolus material. Results are shown in Table III. The extracts used

TABLE III

HYDROLYSIS BY EMULSIN OF ORGANIC CHLORINE COMPOUND FORMED BY POTATO
TISSUE FROM ABSORBED ETHYLENE CHLORHYDRIN

Time, hours	Volatile Cl compd. recovered, millimols			% of org. chlorine recovered	Reducing sugar, as glucose, millimols		
	With emulsin	Without emulsin	Diff.		With emulsin	Without emulsin	Diff.
42.0	0.22	None	0.22	34.8	0.91	0.69	0.22
46.3	0.31	None	0.31	76.0	0.37	0	0.37
41.0	0.27	Trace	0.27	60.0	0.58	0.14	0.44

were obtained as follows. Expressed juice after allowing the starch to settle out was heated to 80° C. and filtered. The filtrate was evaporated to about one-fourth its original volume under vacuum. Upon the addition of a large volume of acetone an oily layer separated out. The acetone layer which contained most of the organic chlorine compound was removed and the acetone distilled off under vacuum and the aqueous solution remaining then contained most of the organic chlorine originally present. Such a solution was used for the first test shown in Table III. For the last two tests solutions prepared in this way were extracted continuously with butyl alcohol and butyl alcohol extracts thus obtained were used after the addition of water and evaporation of the butyl alcohol.

Optical activity. The method used so successfully by Bourquelot [as quoted by Klein (9, p. 810)] for demonstrating the presence of glucosides in plant extracts and which has led to the isolation of a large number of new glucosides depends upon an increase in reducing sugar by the action of emulsin and a change in optical rotation toward the right. All known plant glucosides which are hydrolyzable by emulsin are laevorotatory and thus hydrolysis of the glucoside results in a decrease in the extent of the laevorotation of the solution. Preliminary tests on changes in optical activity of gladiolus extracts containing the chlorine compound hydrolyzable by emulsin have shown such a change in optical rotation. Thus in one experiment a gladiolus extract containing 5.6 millimols of organic chlorine

per 100 cc. gave an observed rotation in a decimeter tube with a sodium light of −0.36°. After hydrolysis by emulsin for 17.5 hours with the formation of 1.75 millimols per 100 cc. of the volatile chlorine compound and 2.78 millimols of reducing sugar calculated as glucose the observed rotation was +0.08°. The observed rotation of an extract of gladiolus tissue which had not been treated with ethylene chlorhydrin (which represented somewhat less tissue than the extract from treated material and was, therefore, more dilute) was +0.06° in a two decimeter tube and after the action of emulsin resulting in the formation of 0.08 millimols of reducing sugar (as glucose) per 100 cc., the change in rotation was so small as to be within the error of the instrument used for determining optical activity. In another experiment an extract of treated tissue which had been partially purified and concentrated and which contained 14.5 millimols of organic chlorine per 100 cc. the observed rotation in a decimeter tube was −1.54°. After treatment with emulsin resulting in hydrolysis of 52 per cent of the chlorine compound and the formation of 10.5 millimols of reducing sugar per 100 cc. the observed rotation was +0.03°.

DISCUSSION

The formation of a glucoside from the absorbed ethylene chlorhydrin may be regarded as a detoxication. Ethylene chlorhydrin is a very reactive compound. It not only contains very active chlorine but is also a fat solvent and it is unlikely that plant tissue could survive the absorption of appreciable quantities of the chlorhydrin if means were not available for rendering it less active. Through the changes which the chlorhydrin undergoes in potato and gladiolus tissue it loses its character as a fat solvent and preliminary tests (unpublished) indicate that the chlorine in the compound formed from the chlorhydrin is less active than in the original chlorhydrin.

If further work substantiates the evidence already obtained for the formation of a β-glucoside by plant tissue from absorbed ethylene chlorhydrin the result is of considerable interest because it offers a definite case in which a plant utilizes β-glucoside formation for rendering less active an introduced chemical. Ciamician and Ravenna are quoted by Armstrong and Armstrong (1, p. 86) as having published work indicating that maize plants can form glucosides when inoculated with aromatic products, such as saligenin, of the hydrolysis of glucosides. Many of the known aglucons are very reactive substances and it is thought that glucoside formation may serve as a means of rendering less harmful toxic metabolic products (9, p. 808). These results with chlorhydrin indicate that the plant can also form glucosides from substances which are not known aglucons and which are not products of metabolic activity. Thus glucoside formation may serve as a detoxication mechanism which can detoxicate introduced chemicals as well as toxic products of plant metabolism.

It is possible also that the formation of a glucoside from the ethylene chlorhydrin or at least the effect of the chlorhydrin treatments on the glucoside-glucosidase balance may be of direct importance in connection with the dormancy breaking action of the chlorhydrin. Armstrong and Armstrong (1) stress the regulatory action of the glucoside systems in plants.

A study of the reactions brought about by living plant tissue on introduced substances should offer a valuable field of investigation yielding information on the types of reactions plant tissues can bring about. The detoxication mechanisms employed by animal organisms have been given much study and have yielded very interesting results. Such studies have received relatively little attention in plant investigations although data available show that a variety of foreign substances undergo reactions when introduced into plants. Thus potato tissue readily reduces methyl and ethyl disulphide to compounds giving the nitroprusside test (10) and several methods proposed for testing the viability of seeds involve the use of chemicals which undergo change as the result of the vital activities of the seeds (7, 8).

If potato and gladiolus tissue can form a β-glucoside from ethylene chlorhydrin it is probable that glucosides can be formed by plant tissue from other substances not known to occur in plants. It may be possible to obtain glucosides in this way from aglucons which do not lend themselves readily to glucoside formation by the ordinary chemical methods.

SUMMARY

The non-volatile organic chlorine compound formed by potato and gladiolus tissue from absorbed ethylene chlorhydrin is hydrolyzed by emulsin with the formation of a volatile organic chlorine compound (which may be ethylene chlorhydrin) and reducing sugar, or at least a substance or substances which reduce Fehling's solution.

Preliminary tests on the effect of emulsin hydrolysis on optical activity (with gladiolus material) show that the shift in optical activity is toward the right.

These results offer strong evidence that the compound formed from the absorbed chlorhydrin is a β-glucoside and that glucoside formation can be utilized by plant tissue to render less active an introduced chemical.

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A NEW METHOD FOR THE DETERMINATION OF GLUTATHIONE IN TISSUES BASED ON ITS REACTION WITH SULPHUR TO FORM HYDROGEN SULPHIDE[1]

JOHN D. GUTHRIE

It is well known that the iodometric methods commonly used for the determination of glutathione in tissues give values that are much too high. This is due largely to the presence of ascorbic acid which also reacts with iodine, but even if correction is made for this, the possibility remains that there are substances in the tissue other than ascorbic acid or glutathione that reduce iodine in acid solution. A more specific reaction for glutathione is its action with elemental sulphur to yield hydrogen sulphide. This reaction, so far as known, is given only by compounds having an SH group. It was suggested by Guthrie and Wilcoxon (3) that this reaction could be used for the estimation of glutathione. However, it was necessary to use the method empirically, since theoretical recovery of H_2S, calculated from the reaction $2GSH + S \rightarrow GSSG + H_2S$, was not obtained by the procedure used. In one form or another the method has been used for several years to determine the glutathione content of potato tissue. Various improvements have been made from time to time, and recently conditions have been found with which theoretical recovery of H_2S can be obtained from glutathione in metaphosphoric acid solution and almost theoretical recovery from extracts of potato tissue.

METHOD

Development of the method. In certain experiments it was desirable to be able to determine both glutathione and ascorbic acid on the same extract. Since the method previously used of killing the tissue in boiling water and extracting with alcohol proved unsatisfactory for ascorbic acid determinations, the metaphosphoric acid extraction of Fujita and Iwatake (2) was tried. Better recovery of added glutathione from neutralized solutions of metaphosphoric acid were obtained than from the dilute alcohol and phosphate buffer previously used, but when neutralized metaphosphoric acid extracts of potato tissue were tried, the recovery was lower than from pure metaphosphate solutions. This indicated that there was some oxidase or heavy metal in the tissue extracts which destroyed either the glutathione or the hydrogen sulphide. In order to counteract this, the addition of KCN to the extracts was tried. This addition increased the yield and gave

recovery values that approached the theoretical. Other important improvements that followed were making the determination at a controlled temperature of 30° C. and using nitrogen containing a little HCN for aerating the H_2S into the zinc acetate receivers. It was also found that the hydrogen sulphide could be determined by iodometric titration somewhat more conveniently than by the colorimetric method previously used, but the colorimetric method may be useful for certain purposes or where the method is used qualitatively.

Recommended procedure. Cut the tissue into small pieces and drop 50 g. into 50 cc. of 5 per cent freshly dissolved metaphosphoric acid contained in a large mortar. Add 10 g. pure quartz sand (washed with aqua regia) and grind until fine. Add 50 cc. more metaphosphoric acid and 50 cc. H_2O. Pour into centrifuge bottles, allow to stand 20 minutes, and centrifuge. Filter the supernatant liquid through cotton. Add 3 cc. of 1 per cent KCN to 60 cc. of the extract and adjust to pH 6.70 by adding about 2 cc. 20 per cent NaOH. A few drops of bromothymol blue may be used as an aid in adjusting the pH.

Measure 50 cc. of the neutralized extract into a Van Slyke-Cullen aeration tube using a graduated cylinder or a pipette operated by a vacuum pump since vapors of HCN are present above the liquid. Add 10 drops of paraffin oil and 2 cc. of a saturated solution of sulphur in absolute alcohol, connect to a receiving tube containing 25 cc. of 2 per cent zinc acetate, place in a bath at 30° C. and aerate at once. Rubber connections between the reaction tube and the receiver should be reduced by using bent glass tubing. Nitrogen containing a little HCN should be used for the aeration. This is obtained by passing nitrogen through 90 per cent phosphoric acid and then over crude calcium cyanide (cyanogas) distributed in three 20-inch tubes connected in series. It is then distributed to each determination. The aeration should be rapid, about 40 l. per hour and should be done in the hood. This procedure should yield 4 to 6 cc. N/10 HCN per hour.

Stop the aeration after 4 hours and add a known amount of N/1000 or N/200 KIO_3 to the receiver, depending on the amount of glutathione. The cloudiness of the receiver, which is due to ZnS, will serve as a rough guide to the amount of KIO_3 to use. Then add 2 cc. of 1 per cent KI, 10 cc. 1:5 HCl, and 1 cc. of 1 per cent soluble starch to the receiving tube. It is important to add the HCl before the starch. Allow to stand until the zinc cyanide is completely dissolved. This may require an hour. Titrate the excess iodine with N/1000 $Na_2S_2O_3$ until all trace of blue tint is gone. From the amount of H_2S formed, calculate the amount of glutathione from the reaction $2GSH + S \rightarrow GSSG + H_2S$. In the above procedure the aliquot used may be assumed to represent 11.54 g. tissue. For tissues containing large amounts of glutathione, smaller aliquots may be used, adding

water to the reaction tube to make 50 cc. For the determination of the H_2S a colorimetric procedure may also be used. This was employed extensively in the development of the method, but later the volumetric method proved more convenient. All quantitative data reported in this paper were obtained with the volumetric method. The colorimetric procedure is as follows.

Convert the H_2S into methylene blue by adding to the receiving tube 5 cc. of para-amino-dimethylaniline hydrochloride, 100 mg. per 100 cc. of 20 per cent HCl, and 5 cc. of $M/50$ $FeCl_3.6H_2O$ in 1:9 HCl. Allow to stand overnight, wash into a volumetric flask, usually 50 cc., and make to volume. Compare in a colorimeter with standards prepared from known amounts of hydrogen sulphide. Prepare the standards from a $N/1000$ solution of H_2S in dilute NaOH, made by dilution from a concentrated solution prepared by passing H_2S through 100 cc. of $N/1$ NaOH for 5 minutes, adding 10 cc. more of the NaOH and diluting to 1.5 liters. Determine the concentration of this solution by iodometric titration using $N/50$ iodine and $N/100$ $Na_2S_2O_3$ and calculate the amount to use for 1 liter of $N/1000$ H_2S. The series of standards generally used were made by adding 1, 2, 5, and 10 cc. of the $N/1000$ solution to 25 cc. of 2 per cent zinc acetate in 50 cc. flasks, adding 5 cc. of para-amino-dimethylaniline hydrochloride and 5 cc. of ferric chloride and making to volume.

Notes. The large number of experiments which led to the adoption of certain details of the above procedure will not be given in detail, but some of the conclusions are given in the following notes on the method.

1. Although nitrogen is recommended instead of air in the aeration, this is based on a very small difference in favor of the former obtained in some of the experiments. In other experiments nitrogen and air gave about the same values. Aeration with nitrogen is recommended, but air might be used without serious error if nitrogen were not available.

2. A little HCN in the nitrogen used for the aeration has been shown to be necessary for the best results under the conditions given. However, in one experiment in which the temperature was increased to 40° C. satisfactory recovery was obtained using KCN in the extract, but no HCN in the nitrogen. This possibility might be investigated further.

3. All the H_2S is absorbed by one receiving tube, since no test for H_2S could be obtained in a second tube when it was used. Adding a little NaOH to the zinc acetate receivers did not increase the recovery of H_2S.

4. The H_2S was in a free condition at the pH used in the determinations since acidifying the solutions toward the end of the aeration did not liberate any extra H_2S.

5. In experiments with metaphosphoric acid solutions and no KCN or HCN, all of the H_2S came over in about three hours. When KCN and HCN were used, a little H_2S continued to come over even after four hours'

aeration. This was due to the slight reducing action of the HCN on the oxidized glutathione formed in the reaction and appears to be unavoidable. This action appeared to compensate nicely for certain small losses so that four hours at 30° gives values close to theoretical.

6. The effect of the cyanide in the receivers during the determinations of the H₂S has been investigated and found to be insignificant in both the colorimetric and volumetric procedures when these are properly carried out.

7. The receiving tubes may be allowed to stand overnight before determining the H₂S if kept closed.

8. Since experiments showed that better recovery was obtained when the rubber connections between the reaction tube and the receiver were reduced to a minimum by the use of glass tubing, this procedure was adopted.

9. Since it was found that temperature fluctuations affected the yield and that recovery was better at a temperature slightly above that of the laboratory, a constant temperature bath of 30° C. was tried and found satisfactory. Temperatures of 40° and 50° C. were also tried and found to give too high values. This was due to the higher temperature increasing the reducing action of HCN on the oxidized glutathione formed in the reaction. Attempts to eliminate this by decreasing the pH or shortening the aeration time did not lead to procedures as satisfactory as the one outlined.

10. The amount of sulphur used in the method is adequate up to at least 8 mg. glutathione. This was shown by calculations and also by adding more in certain experiments, either at the start or at intervals. The saturated solution of sulphur in alcohol is made by heating absolute alcohol with an excess of powdered sulphur, allowing to cool, and filtering.

Action of HCN. It was found necessary to use HCN in the method in order to eliminate the action of oxidase and heavy metals in the tissue extracts. Attempts to substitute some other reagent have failed. KSCN, thiourea, pyrophosphate, and ethyl carbylamine, the ethyl ester of HCN, have been tried. Ethyl carbylamine, C_2H_5NC, which was prepared and tried because Toda (6) found it inhibited catalysis by heavy metals, completely inhibited the reaction, probably by combining with the H₂S. The chief objection to the use of HCN is that under favorable conditions it reduces oxidized glutathione to reduced glutathione which could then react with sulphur. Under the conditions of the method this action is reduced to a minimum by choosing the correct pH, since the reduction of oxidized glutathione by HCN is favored by an alkaline reaction. Since it seems to be the consensus of opinion that extracts of living tissue properly made contain very little or no oxidized glutathione, the reducing action of HCN would be limited to the oxidized glutathione formed in the re-

action. This unquestionably takes place to a slight extent and should lead to high results if it were not compensated by certain minor losses in the procedure. The use of HCN does, however, make mandatory that the temperature, pH, and time of aeration be controlled in order to obtain the best results.

The necessity of using HCN with tissue extracts to obtain good recovery is illustrated by the following: metaphosphoric acid extracts of potato tubers and of guinea pig livers were prepared in the usual way, adjusted to pH 6.70, a saturated solution of sulphur added and aerated with nitrogen for several hours to oxidize any glutathione present. Part of each extract was then slightly acidified, the usual amount of KCN added and adjusted again to pH 6.70. The recovery of added glutathione from these extracts, using N_2 containing HCN, was compared with that from the extracts containing no KCN and aerated with nitrogen. Without KCN or HCN 0.45 mg. was recovered from 1 mg. of added glutathione in the case of potato extract and 1.35 mg. from 2 mg. in the case of liver extract. With KCN the values found were 0.99 mg. with the potato extract after subtracting a blank of 0.38 mg. and 2.16 mg. with the liver extract after subtracting a blank of 1.00 mg. A number of other experiments also showed clearly that HCN must be used and considerable work was done in establishing the amount of KCN to use and the method of obtaining nitrogen containing a little HCN.

In order to see what the magnitude of the action of HCN on oxidized glutathione would be, 5 mg. portions of oxidized glutathione prepared according to Mason (5) were tried in several determinations using metaphosphate solutions containing KCN at various pH values and following the method in all respects except for pH. The results are given by the following.

pH:	6.25	6.44	6.68	6.86	7.27	7.83
GSH found:	0.00	0.07	0.14	0.26	0.93	1.29

It will be seen that even with so large an amount of oxidized glutathione as 5 mg., it is equivalent in the method at 30° C. to only 0.14 to 0.26 mg. reduced glutathione. From 5 mg. oxidized glutathione a value of 0.45 mg. was obtained at 40° C. and 1.61 mg. at 50° C. at pH 6.70.

Choice of pH. Several experiments in which the pH was varied were made during the development of the method. Most of these data will not be given since they were obtained prior to certain important changes in the procedure. Glutathione begins to react with sulphur at about pH 5.0 and theoretical recovery under the conditions of the method are obtained at about pH 6.70. At values much over pH 7.0 high values may result from the action of the HCN on oxidized glutathione which is favored by increased alkalinity. In one experiment with various pH values, but other-

wise closely following the method, amounts of 1.81, 1.90, 2.02, and 2.06 mg. were obtained from 2 mg. glutathione in metaphosphate solutions at pH 6.63, 6.80, 6.90, and 7.02 respectively. Since in later experiments at pH 6.80 the reaction sometimes shifted to about pH 6.90 during the aeration with the result that slightly high values were obtained with the higher amounts of added glutathione, it was decided to recommend pH 6.70 in the method.

SPECIFICITY OF THE METHOD

The sulphur method has been tried on a number of compounds and so far only compounds having an SH group have given strong reactions. Weak tests were obtained with compounds having a disulphide group due to a slight reducing action of the HCN used in the method to yield the corresponding SH compound. Presumably if HCN were omitted, disulphide compounds would be negative. All other compounds tested besides sulfhydryl compounds or disulphide compounds have been negative. The following compounds gave no H_2S in the sulphur method using the colorimetric procedure: ergothioneine, dl-methionine, thiourea, potassium thiocyanate, thioacetamide, thiobarbituric acid, dl-valine, l-leucine, lysine dihydrochloride, l-hydroxyproline, p-hydroxyphenyl glycine, glycine, glutamic acid, arginine, tryptophane, alanine, phenylalanine, l-tyrosine, dl-α-amino-N-butyric acid, l-proline, aspartic acid, ascorbic acid, cinnamic acid, citric acid, l-malic acid, tartaric acid, oxalic acid, succinic acid, benzoic acid, fructose, glucose, sucrose, creatine, creatinine, hypoxanthine, uric acid, adenine sulfate, and urea. Positive results were obtained with cysteine hydrochloride, thiosalicylic acid, thioglycolic acid, ethyl mercaptan, and butyl mercaptan. Faint reactions were obtained with oxidized glutathione, l-cystine, and methyl disulphide.

The ergothioneine used in the above tests was generously supplied by Dr. George Hunter of the University of Alberta. Its failure to react with sulphur to form H_2S may indicate that it has the formula:

$$CH = C.CH_2.CHCO$$
$$| \quad | \qquad | \quad |$$
$$HN \ NH \qquad N - O$$
$$\diagdown \diagup$$
$$C \qquad (CH_3)_3$$
$$\|$$
$$S$$
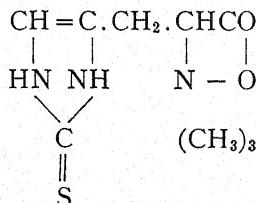
instead of the sulfhydryl formula usually given. Hunter and Eagles (4) have shown that the sulphur in ergothioneine is much more firmly bound than sulphur in glutathione.

RECOVERY OF ADDED GLUTATHIONE FROM METAPHOSPHATE SOLUTIONS AND FROM TISSUE EXTRACTS

In order to test the method, recovery from various amounts of glutathione added to metaphosphate solutions and to tissue extracts was determined. In the case of the metaphosphate solutions the procedure was the same as in the method, starting with 2.5 per cent metaphosphoric acid, adding KCN, and adjusting to the required pH. The tissue extracts were made in the way described in the method, but received a preliminary treatment with sulphur to oxidize most of the glutathione present and thus
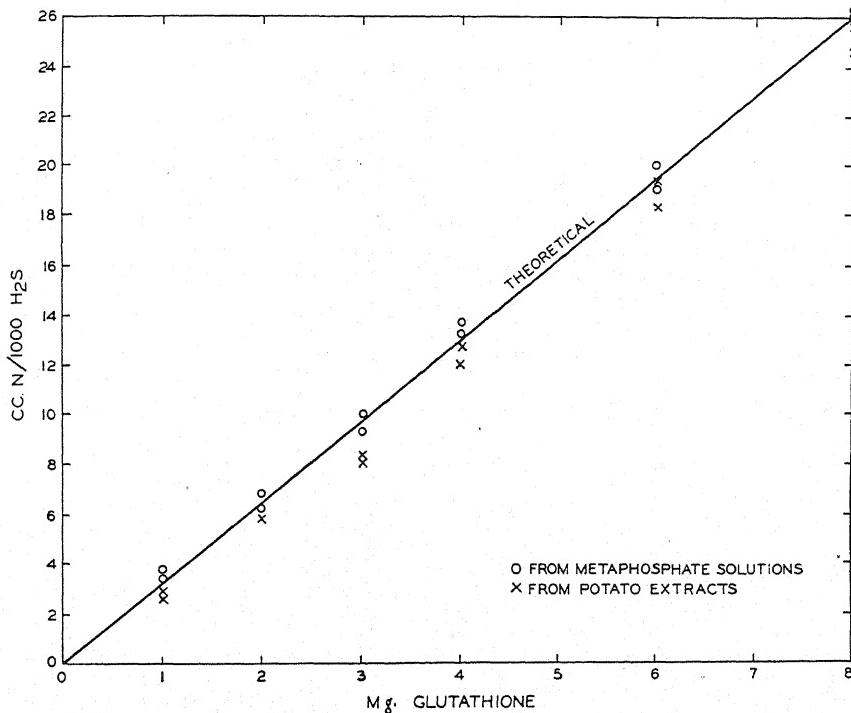
FIGURE 1. The production of H_2S from various amounts of glutathione in the sulphur method applied to metaphosphate solutions and to metaphosphoric acid extracts of potato tissue. The theoretical line is calculated from the reaction $2GSH + S \rightarrow GSSG + H_2S$.

reduce the blank. They were adjusted to pH 6.7, 10 cc. of a saturated solution of sulphur in absolute alcohol added to 420 cc. of extract, and aerated with nitrogen for four hours. Potassium cyanide, 21 cc. of 1 per cent, was then added and the pH adjusted again to pH 6.70. Various amounts of glutathione, 10 drops of paraffin oil, and 2 cc. of a saturated solution of absolute alcohol were added to 50 cc. portions of this solution and the aeration started at once according to the procedure outlined in

the method. In the case of guinea pig liver extracts, 25 cc. of the extract and 25 cc. of water were used. Blanks averaging 0.35 mg. glutathione were obtained with the potato extracts and blanks of 1.00 mg. for the liver extract. These were subtracted from the values found with various amounts of added glutathione. Some of the results are shown in Figure 1 where the cc. of N/1000 H_2S obtained are plotted against the amounts of glutathione added. It will be seen that amounts closely agreeing with the theoretical were obtained with metaphosphate solutions. With potato extracts, the yields were slightly lower, but averaged 90 per cent of the expected. Other results are given in Table I. In this series of experiments the average recovery from metaphosphate solutions was 103 per cent, and from potato extracts 92 per cent. The average recovery from the liver extract was 104 per cent.

TABLE I

RECOVERY OF ADDED GLUTATHIONE FROM METAPHOSPHATE SOLUTIONS, FROM EXTRACTS OF POTATO TISSUE AND FROM EXTRACTS OF GUINEA PIG LIVER BY THE SULPHUR METHOD

Glutathione added, mg.	Glutathione found, mg.				
	Metaphosphate solution		Potato extract		Liver extract
	pH 6.80	pH 6.70	pH 6.70	pH 6.68	pH 6.70
1.00	1.04		0.88	0.83	0.96
2.00	2.02	2.13	1.89	1.63	2.16
3.00	3.07	2.85	2.81	2.58	2.88
4.00	4.30	4.05	4.03	3.55	4.35
6.00	6.30	6.02	6.05	5.36	6.75
8.00	8.32	8.42	8.13	7.41	8.12

In order to test the method further, known amounts of glutathione were added to potato tissue just prior to analysis and the recovery determined. To 50 g. tissue containing 8.5 mg. glutathione, 20 mg. glutathione were added. The glutathione found was 27.3 mg. or a recovery of 94 per cent. Similarly, 10 mg. were added to the same tissue and 18.0 mg. found or a recovery of 95 per cent.

A COMPARISON OF THE METHOD WITH OTHER METHODS FOR GLUTATHIONE

A comparison of the method was made with some of the methods frequently used for the determination of glutathione. The first procedure was to add 2 cc. of 5 per cent KI to 25 cc. of the metaphosphoric acid extract and titrate with N/100 KIO_3 using starch as an indicator. Since this is known to give high results in the presence of ascorbic acid, another aliquot of the extract was titrated with 2,6-dichlorophenolindophenol in order that a correction could be made for ascorbic acid. Some of the results are given in Table II where the two iodometric procedures are compared with the sulphur method on potato and guinea pig liver tissue.

In some cases the potato tissue was from tubers treated with ethylene chlorhydrin, a treatment which greatly increases their glutathione content. The guinea pig liver tissue was kindly supplied by Mr. Setterstrom and Dr. Hartzell of this Institute. Two of the guinea pigs had been in an atmosphere of 10 p.p.m. SO₂ for 30 days. It will be seen that very high results are obtained by simple iodometric titration and that even if this

TABLE II

A COMPARISON OF VARIOUS METHODS FOR GLUTATHIONE USING POTATO AND LIVER TISSUE

Tissue used	Glutathione, mg. per 100 g.		
	Iodometric titration	I titration less ascorbic acid	Sulphur method
Potato treated with CH_2ClCH_2OH	226	112	63.2
Potato untreated	81	15	9.8
Potato treated with CH_2ClCH_2OH	135	52	31.9
Potato untreated	76	15	8.9
Liver from guinea pig, SO₂ 10 p.p.m.	421	369	198
Liver from guinea pig, SO₂ 10 p.p.m.	347	300	116
Liver from guinea pig, control	421	376	183
Liver from guinea pig, control	515	462	221

is corrected for ascorbic acid the results are much higher than obtained by the sulphur method. Because of the greater specificity of the sulphur method, the above results are interpreted as indicating that both potato and liver contain iodine-reducing substances other than ascorbic acid and glutathione.

TABLE III

A COMPARISON OF VARIOUS METHODS FOR GLUTATHIONE USING POTATO TISSUE

Treatment of tubers	Glutathione, mg. per 100 g.			
	Iodometric titration	I titration less ascorbic acid	Binet and Weller method	Sulphur method
Treated with CH_2ClCH_2OH	115	58	41.2	40.2
Untreated	54	16	9.5	7.6
Treated with CH_2ClCH_2OH	181	94	59.0	60.8
Untreated	59	12	8.6	9.6

Other results of comparison of methods are given in Table III. In this case the method of Binet and Weller (1) was also used. This method depends on a precipitation of glutathione with cadmium lactate to obtain specificity prior to iodometric titration. The results show that with potato tissue it gives results in good agreement with those obtained by the sulphur method.

SUMMARY

A method for the determination of glutathione in tissues based on the reaction $2GSH+S \rightarrow GSSG+H_2S$ is described. The method gives satisfactory recovery of added glutathione from tissues and from tissue extracts. Due to its specificity it gives lower results than the iodometric method, even when this is corrected for ascorbic acid. It is in good agreement with the cadmium lactate method of Binet and Weller when applied to potato tissue.

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THE UTILIZATION OF SULPHATE IN THE SYNTHESIS OF GLUTATHIONE BY POTATO TUBERS FOLLOWING TREATMENT WITH ETHYLENE CHLOROHYDRIN[1]

John D. Guthrie

A large increase in the glutathione content of potato tubers takes place following treatment with ethylene chlorohydrin (4) and a decrease in the sulphate content of the juice of the tubers also occurs (5). A connection between these two changes has been pointed out before (5,6), but detailed publication of the experiments bearing on this has awaited the development of a method for glutathione (7) that would give values close to the true ones and not merely relative values, since it seemed important to establish definitely the quantitative relation between the decrease in sulphate and the increase in glutathione.

EXPERIMENTS

Some of the results of earlier experiments dealing with the quantitative relation between the increase in glutathione and the decrease in sulphate in potato tissue (*Solanum tuberosum* L.) are shown in Table I. These data were obtained by the sulphur method for glutathione applied to hot water-

TABLE I

RELATION BETWEEN INCREASE IN GLUTATHIONE AND DECREASE IN SULPHATE IN POTATO TUBERS FOLLOWING TREATMENT WITH ETHYLENE CHLOROHYDRIN

Source of tubers	Glutathione by sulphur method, cc. M/100 per 100 g.		Sulphate, cc. M/100 per 100 cc.		Increase in glutathione	Decrease in sulphate
	Treated	Check	Treated	Check		
S. Carolina	16.4	3.1	14.3	37.3	13.3	23.0
S. Carolina	16.2	3.8	18.2	38.9	12.4	20.7
Maine	12.7	2.1	44.2	63.0	10.6	18.8

alcohol extracts of the tissue and are corrected for recovery. With the procedure then in use the recovery from known amounts of glutathione added to the same extracts was determined and the glutathione values corrected with this value. Since the hydrogen sulphide obtained was about 50 per cent of the theoretical expectations, the correction was quite large and consequently the absolute value for the glutathione increase was open to question. Sulphate was determined on the boiled, filtered juice by precipitation as barium sulphate in the presence of hydrochloric acid. Following the ignition of the precipitate it was washed with hydrochloric acid

and weighed again. The details of this procedure will be found elsewhere (5). Since a mole of sulphate is equivalent in sulphur content to a mole of glutathione, all values are reported in terms of cc. M/100 per 100 g. tissue or 100 cc. of juice. For soluble constituents of tissues with high water content like potato tubers, 100 cc. of juice may be considered equivalent to 100 g. of fresh tissue for all practical purposes [see Sayre and Morris (10)]. The tubers used for the experiments reported in Table I were Irish
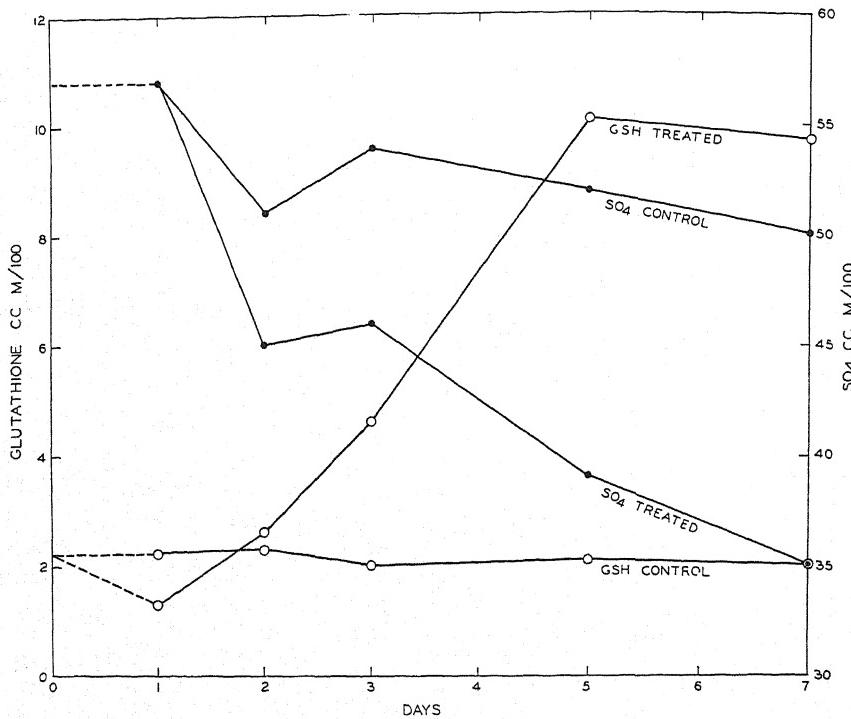
FIGURE 1. The sulphate and glutathione content of tubers treated with ethylene chlorohydrin and of untreated tubers at different times after treatment.

Cobbler variety from South Carolina and from Maine. Cut pieces were treated with ethylene chlorohydrin, 40 cc. of 40 per cent per liter, for 24 hours by the dip method of Denny (3). The check samples were similarly treated with water. They were planted for 5 days prior to analysis. It will be seen that in every case a large increase in glutathione took place and also a decrease in sulphate. The decrease in sulphate was more than equivalent to the increase in glutathione. The ratio of the glutathione increase to the sulphate decrease averaged 0.6 in these experiments, but as pointed out above, the actual magnitude of the glutathione increase was somewhat uncertain.

In order to test further the connection between the increase in glutathione and the decrease in sulphate, it was desirable to see if they occurred at the same time following treatment with ethylene chlorohydrin. Cut pieces were treated by the dip method and analyzed for glutathione and sulphate at 1, 2, 3, 5, and 7 days after the start of the treatment. The results are shown in Figure 1. It will be seen that the decrease in sulphate took place at about the same time as the increase in glutathione. In an experiment with whole tubers treated by the vapor method, glutathione and sulphate had not changed significantly 17 hours after the start of the treatment, but at 40 hours an increase in glutathione and a decrease in sulphate was found. In another experiment neither change had occurred at 13 hours, but both changes were found at 60 hours.

In an experiment made for another purpose, some Bliss Triumph tubers from Cuba were treated with ethylene chlorohydrin by the vapor method, 4 cc. of 40 per cent per kg. for 24 hours, and then stored in oxygen. For some reason this particular lot of tubers was very slow in giving an increase in glutathione when stored whole. The glutathione increase had not taken place when they were analyzed four days after treatment, although pH of the juice had changed from pH 5.83 to pH 6.90 and the treated sample was very active in reducing methylene blue. Thus, some of the changes that usually take place in potato tubers treated with ethylene chlorohydrin had occurred, but for some reason the glutathione increase had not. The juice was analyzed for sulphate. The results are given in the first experiment of Table II. No significant change had taken place in the sulphate content. This shows that the sulphate decrease is not closely connected with the change in pH or the reducing power of the juice, but is dependent on the glutathione increase. All the glutathione data in Table II were obtained by the improved sulphur method (7) which has been shown to give correct values for glutathione. Values obtained by titrating metaphosphoric acid extracts of the tissue iodometrically and also with 2,6-dichlorophenolindophenol so that correction could be made for ascorbic acid are reported. This quantity is called non-ascorbic acid reducing substances and includes glutathione as well as other substances that react with iodine in acid solution. These values are much higher than the values found by the more specific sulphur method. They are given because they unquestionably represent maximal values and since they show much the same thing as the glutathione values by the sulphur method, should satisfy those who prefer the old iodometric procedures for glutathione.

In certain experiments it was found that the glutathione increase was hastened by cutting the tubers into pieces following treatment with ethylene chlorohydrin by the vapor method. It was evident that a crucial test of the idea that sulphate was used in the synthesis of glutathione could be made in this way, since if this concept were true, the sulphate

decrease should likewise be hastened by cutting the tubers. As shown in the second and third experiments of Table II, this proved to be the case. In both experiments the tubers were treated with ethylene chlorohydrin by the vapor method using 4 cc. of 40 per cent per kg. for 24 hours. The

TABLE II

INCREASE IN GLUTATHIONE AND DECREASE IN SULPHATE IN POTATO TUBERS UNDER VARIOUS CONDITIONS

Tubers used	Condition of tubers after treatment	Non-ascorbic acid reducing substances, cc. M/100 per 100 g.		Glutathione by sulphur method, cc. M/100 per 100 g.		Sulphate, cc. M/100 per 100 cc.	
		Treated	Check	Treated	Check	Treated	Check
Bliss Triumph from Cuba	Whole, kept in O_2	7.6	8.5	3.2	3.9	46.4	47.5
Bliss Triumph from Institute garden	Whole Cut and planted	12.0 36.6	5.6 5.0	7.7 20.6	3.5 3.2	29.4 1.1	42.2 44.5
Irish Cobbler from S. Car.	Whole Cut, in moist chamber	11.5 16.7	9.9 9.5	5.8 9.6	5.0 3.7	36.1 20.4	48.5 48.1

controls were kept in a closed container for 24 hours. In one experiment part of the tubers were cut up, planted, and analyzed four days after the start of treatment. It will be seen that at this time a much larger decrease in sulphate was found in the cut pieces than in the whole tubers. In fact in the cut pieces the sulphate had almost disappeared from the treated pieces. In the other experiment, the cut pieces were placed in moist cham-

TABLE III

QUANTITATIVE RELATION BETWEEN INCREASE IN GLUTATHIONE AND DECREASE IN SULPHATE IN POTATO TUBERS FOLLOWING TREATMENT WITH ETHYLENE CHLOROHYDRIN

Treatment	Non-ascorbic acid reducing substances, cc. M/100	Glutathione, cc. M/100			Sulphate, cc. M/100 per 100 cc. juice
		B. & W. method		Sulphur method	
		Juice	Tissue		
Ethylene chlorohydrin Control, closed container	30.5 3.9	20.8 2.0	19.2 2.8	19.8 3.2	16.5 62.0
Change	26.6	18.8	16.4	16.6	45.5

bers instead of being planted. The analysis was made 5 days after the start of treatment. It will be seen that a larger increase in glutathione and a larger decrease in sulphate took place in the cut pieces than in the whole tubers.

As additional evidence to evaluate the quantitative relation between glutathione and sulphate, an experiment was made in which glutathione

was also determined by the method of Binet and Weller (1), in which specificity is obtained by a cadmium lactate precipitation prior to iodometric titration. A determination was also made on the boiled, filtered juice by this method modified to include oxidized glutathione (2). Non-ascorbic iodine reducing substances were also determined. The results given in Table III show that essentially the same results are obtained with the Binet and Weller method as with the sulphur method. The ratio of the glutathione increase to the sulphate decrease averages 0.38 in this experiment. The ratio of the increase in non-ascorbic acid reducing substances, which includes glutathione, to the sulphate decrease is 0.58.

DISCUSSION

Some of the steps leading to the formation of glutathione following treatment with ethylene chlorohydrin are now known. The first observed action is a large increase in respiration (9). Citric acid acts as a substrate for this increased respiration (5) and is converted to carbon dioxide. As a result of this loss of citric acid the tissue becomes less acid and the increase in glutathione is correlated with this decreased acidity. Sulphate is utilized in the synthesis of the newly formed glutathione and consequently the sulphate content of the tissue decreases. Hammett and Reynolds (8) have recently reported the formation of disulphide from sulphate by an enzyme present in bean root tips. However, much more than a simple enzymatic reaction must be involved in the building up of glutathione from sulphate, although such an enzyme as described by Hammett and Reynolds may be active at some step in the process.

The fact that the decrease in sulphate sulphur is considerably larger than the increase in glutathione sulphur shows clearly that the sulphate is also used in the formation of some sulphur compound besides glutathione. It is hoped that further work will lead to the identification of this compound.

SUMMARY

Sulphate sulphur is utilized by potato tissue in the synthesis of glutathione that follows treatment of the tubers with ethylene chlorohydrin. A quantitative investigation of this process shows that part of the sulphate sulphur enters a compound other than glutathione.

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ORIENTATION IN YOUNG COTTON FIBERS AS INDI-
CATED BY X-RAY DIFFRACTION STUDIES

Wayne A. Sisson[1]

The mature cotton fiber possesses a variable orientation with reference to the fiber axis which can be estimated from either microscopic (4) or X-ray diffraction studies (12, 18). The relationship of crystallite orientation to the arrangement of certain microscopically visible configurations in the cell wall (4), and the correlation between orientation and certain physical properties (15), make it of both theoretical and practical interest to know at what period the X-ray orientation develops during the approximately 50-day duration of cotton fiber growth.

The usual methods of determining orientation consist of either a microscopic study of the visible fibrils, striations and cracks in ordinary light; the study of extinction angles, of dichroism and other phenomena in polarized light; or the study of X-ray diagrams. From the X-ray diagrams, the degree of preferred orientation is indicated by the extent to which the diffraction rings are concentrated into arcs. If the orientation is random, the diffraction rings exist as continuous circles; if it is parallel, the rings are concentrated into arcs. In Figure 1 A, the width of the X-ray arc is a measure of the orientation with reference to the cotton fiber axis. A comparison of Figures 1 A and 1 B shows that the submicroscopic crystalline structure revealed by X-rays usually runs parallel to visible configurations such as fibrils. The usual X-ray diagram, however, represents the average orientation throughout a millimeter section of several thousand fibers, while microscopic results are specific for localized sections of single fibers. Large numbers of microscopic observations are needed to show the structural variations which occur from fiber to fiber and from base to tip of the same fiber. Furthermore, the X-ray pattern registers not only the visible, but also the submicroscopic orientation. The principal value of the X-ray data, therefore, lies in the fact that it gives a general or average value, which can be estimated quantitatively (18). In addition, since each constituent gives its own characteristic diffraction pattern, X-rays may be used in some cases to study the relative orientation of two or more constituents, such as wax and cellulose, as they occur together in the cell wall.

The first X-ray examination of young cotton fibers was made by Clark, Pickett, and Farr (1) on four samples, 18, 21, 35, and 50 days old. The earliest evidence of orientation was found in the 35-day sample. Hess, Trogus, and Wergin (8), in their examination of young cotton fibers, made no special attempt to determine the age at which orientation developed,

but their published diagrams show the first appearance at 40 days. Wergin (19) reported two crystalline constituents to be present during the growth of the cotton hair; the first [later identified as wax (6)] is not oriented, while the second (cellulose) appears as an oriented diagram on the thirty-fifth day.

The present study includes not only the orientation of cellulose at various stages of cell wall development, but also the production of artificial orientation by stretching, the orientation of noncellulosic constitu-
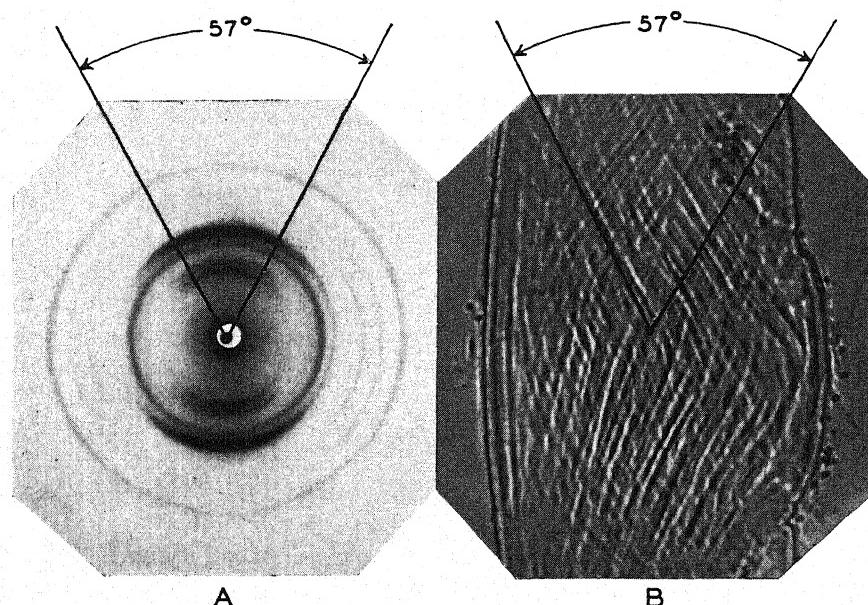
FIGURE I. Comparison of X-ray and microscopic orientation: (A) X-ray diffraction pattern of mature cotton, (B) Photomicrograph of cotton fiber slightly swollen in phosphoric acid to bring out fibril structure.

ents present, and the relative orientation of cellulose in mature fibers of different varieties. This investigation was carried out in connection with previously reported work (16) on the X-ray identification of cellulose in young cotton fibers.

MATERIALS AND METHODS

Many of the samples were prepared from the same material examined in the earlier work (16) which contained the varieties Super Seven and Acala (*Gossypium hirsutum* L.). The material for the present investigation also included the additional variety Pima (*Gossypium barbadense* L.), which was grown under greenhouse conditions (3) during the summer of 1937. The flowers were tagged, the samples collected, and all chemical treatments and X-ray diagrams made as previously described (16), with

the exception that the fibers for X-ray examination were arranged in parallel bundles instead of in random arrangement.

The parallel bundles were prepared by carefully dissecting from the seed a few fibers which were first arranged parallel, and then transferred to a glass plate. Parallel alignment of the fibers was confirmed by microscopic examination. A number of small bundles of parallel fibers were thus built up and allowed to dry. The final, larger bundle consisted of several smaller bundles wrapped together in a special wrapping device[2] with a 2–3 mm. unwrapped section at the center. In making the diffraction patterns, the X-ray beam passed through the unwrapped section of the bundle. Each bundle was examined as originally prepared, and again after subsequent extractions with chloroform and one per cent sodium hydroxide.

The fragility and short length of the younger fibers made it impossible to prepare bundles of parallel fibers younger than 15 days by the above method. Earlier than 15 days, parallel bundles smaller than those described above were built up by placing fibers over the open end of a glass tube. Upon drying, the fiber ends adhered to the glass, producing in the bundle a tension which helped to parallel the fibers. A similar technique was employed in preparing some of the samples later discussed under the heading of induced orientation. A few samples, including fibers both older and younger than 15 days, were prepared in the form of thin films, which were examined with the X-ray beam both perpendicular and parallel to the surface.

The degree of orientation was estimated quantitatively by measuring with a densitometer the density distribution around the 002 diffraction ring, as described in detail elsewhere (15, 18). Owing to the difficulties encountered in arranging fibers of different ages in comparable parallel bundles, the comparison of orientation at different ages is not as accurate as in different samples of mature cotton (15). This is especially true for the younger extracted fibers where the original orientation may be disturbed by the extraction process.

RESULTS

The data obtained on a total of approximately 150 samples for the three varieties may be summarized as follows:

Cellulose orientation. The X-ray patterns of cotton fibers younger than about 25 days show a random orientation. Between 25 and 30 days there develops a definite preferred orientation, the degree of which increases until approximately the thirty-fifth day, after which there is little or no change in orientation. X-ray diagrams illustrating the various stages in

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[2] The wrapping device is similar to that described by Richardson, Bailey, and Conrad (10), with the exception that it is smaller; the fibers are held under less tension, and are wrapped with a single silk fiber instead of cotton thread.
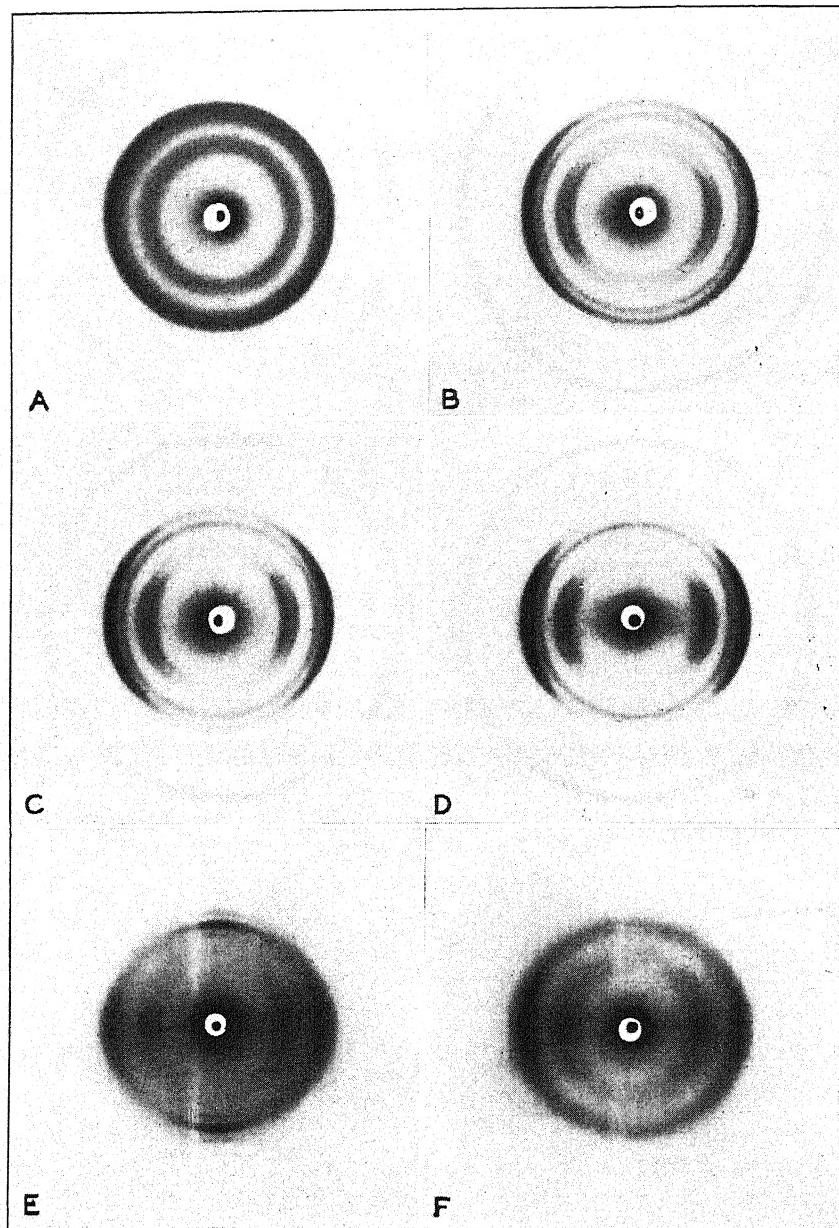
FIGURE 2. Representative X-ray diffraction patterns showing: First, natural orientation at different ages; (A) 15-day, (B) 25-day, (C) 30-day, and (D) mature bundles of parallel fibers extracted in chloroform and sodium hydroxide. Second, induced orientation; (E) stretched film of 20-day fibers after treatment with two per cent sodium hypochlorite, showing orientation of both wax and cellulose lines, and (F) same sample as (E) after further extraction in chloroform.

the development of orientation are shown in Figure 2 A, B, C, and D.

In order to determine definitely the degree of cellulose orientation in the samples between 25 and 35 days, it was necessary, in most cases, to remove the superimposed wax pattern by extracting the fibers with chloroform. The superimposed amorphous diagram in many of the younger fibers (15 to 25 days) made it necessary to extract further with sodium hydroxide to obtain a definite cellulose pattern. The failure to remove these interfering noncellulosic constituents may account in part for the later date at which other investigators (8, 19) find the first appearance of oriented cellulose.

Since the samples were grown in a greenhouse at Yonkers, New York (latitude 41°), it is possible that the present fibers may differ from those grown under field conditions or in warmer climates. Furthermore, owing to the irregularities of growth previously noted (16), it is impossible to state the exact age at which a particular stage of development is reached. All the changes in X-ray orientation are gradual and the results on different bolls do not on the average check closer than three to ten days. For example, in one boll, the orientation at 25 days appeared to be as fully developed as in another at 40 days; in some bolls the orientation was present at 20 days, in others not until after 35 days. This general variation of the X-ray pattern from boll to boll is in agreement with specific microscopic variations of both young (2) and mature fibers (4). The age reported for each stage of development represents that which occurred most frequently.

The X-ray orientation apparently arises from the spiral fibril structure of the secondary wall. The fact that orientation is not present in the X-ray diagram of cotton younger than approximately 25 days, however, does not mean that a spiral fibril structure is not present earlier in localized sections of some fibers. It means that the major part of the cellulose at the middle of the fiber bundle does not have a preferred orientation with reference to the fiber axis. Furthermore, owing to the difficulty of obtaining a bundle of strictly parallel fibers, the natural orientation of which may be disrupted while preparing the sample, it is to be expected that X-ray orientation would not appear until a pronounced coherent spiral structure had begun to form. In general, the first appearance of orientation in the X-ray diagram coincides with that period of fiber development in which there is a decrease in the rate of fiber elongation and an increase in the rate of cell wall thickening.

The increase in orientation with age may be due either to an increase in the ratio of oriented to nonoriented cellulose, or to the cellulose being deposited with a steeper spiral angle as the cell wall thickens. The first possibility appears to be the major factor as indicated by the fact that the orientation approaches a more or less constant value as the cell wall

reaches maturity. Furthermore, it is in agreement with the microscopic observation that in the younger stages of development there are in the lumen large numbers of cellulose particles (5) with random orientation; as the wall thickens, successive oriented lamellae are formed, the lumen narrows and the proportion of random particles decreases.

The absence of any appreciable change in orientation after approximately the thirty-fifth day is in agreement with the findings of Preston (9) that new cellulose deposited in the wall of wood cells assumes an orientation similar to that already present. In wood, however, the cellulose X-ray pattern becomes oriented at an earlier period (10 days) than in cotton (11).

The orientation previously discussed refers to the alignment of the *b* axes of the crystallites (i.e., the direction of cellulose chains which are parallel to the fibrils) with reference to the fiber axis. As discussed in more detail elsewhere (12), there is also the possibility of a preferred orientation of a further crystallographic axis perpendicular to the surface of the cell wall (selective orientation). No definite evidence of a selective orientation, however, was found in the young cotton fibers, which is in agreement with earlier work on mature cotton (12). Since selective orientation usually results from dehydration under special conditions (13, 14), it is possible that cotton fibers are not formed under conditions favorable for the production of selective orientation—a possibility which is being investigated further.

Orientation of noncellulosic constituents. Since noncellulosic materials are closely associated with and greatly affect the behavior of cellulose, it is of special interest to know if they, like cellulose, are oriented in the cotton fiber. Some of these noncellulosic constituents give an amorphous, others a crystalline pattern. The amorphous pattern present in the X-ray diagram of young cotton fibers shows a random orientation at all times. The crystalline materials, with the exception of waxes, are not present in sufficient quantities to be studied as they exist in the fiber.

The wax pattern, reported by Wergin (19) to possess a random orientation, was found in the present work to show a preferred orientation under certain conditions. It is almost always oriented when the sample is prepared in the form of a thin film, and occasionally in a bundle; especially in fibers between 20 and 35 days, or when the bundle is dried under tension. The orientation of both wax and cellulose in the same sample is illustrated in Figure 2 E, where the wax lines are present as arcs at the poles and the cellulose lines at the equator. The oriented wax diffraction lines which arise from side spacings of the wax molecules show intensity maxima at right angles to the corresponding side spacings of cellulose. This indicates that the wax molecules are oriented perpendicular to the cellulose molecules. Waxes are thought to be concentrated in the cuticle,

but it is not possible to determine from the present X-ray data whether the wax is oriented perpendicular to the surface of the fiber (cuticle) or the surface of the fibrils. If the waxes are extracted from cotton and carefully prepared as a thin film on a glass or chromium surface, they may show an oriented X-ray diagram. A detailed X-ray study of the extracted waxes has been made by Gundermann, Wergin, and Hess (6).

Pectic acid can be isolated from young cotton fibers (7) and prepared in the form of films which show orientation (17). Whether or not it is oriented in the fiber, however, is difficult to determine, since pectin gives diffraction lines upon which are superimposed those of the cellulose, waxes, and other constituents of the fiber.

In a few of the younger (10–20 day) samples examined, there is present, as two sharp arcs on the equator of the X-ray diagram, a diffraction line which corresponds to the approximate spacing of 14.5Å. The material which gives this oriented pattern has not been identified.

Experimentally induced orientation. It is well known that orientation can be induced in swollen cellulosic materials by stretching or permitting them to dry under special restrictions. By employing methods successfully applied to other materials (13, 14), attempts were made to induce orientation in young cotton fibers. There was no appreciable change in orientation when unextracted and chloroform extracted fibers, originally showing a random orientation, were stretched and allowed to dry under tension. If they were further extracted in a one per cent sodium hydroxide solution, they showed, in some cases, a slight preferred orientation. When, however, the above treatments were applied to more mature fibers which already showed orientation, then the existing orientation was greatly improved, especially after extraction with sodium hydroxide.

Oriented samples can be produced from very young fibers if, in addition to extraction, they are treated with two per cent sodium hypochlorite to the extent that the original fiber membrane is partially broken down and the resulting cellulose product bleached white. By stretching this purified material or allowing it to dry as a film, it was possible to produce orientation in samples obtained from fibers ranging in age from 6 days to maturity, as illustrated in Figure 2 F. In preparing the sample, it is important that the purification process does not destroy the gelatinous nature of the cellulose residue. To produce orientation, the residue must dry to form a coherent membrane, rather than a granular mass which is easily reduced to a powder.

The failure to produce orientation by stretching very young cotton fibers is probably due to the fact that the small amount of cellulose present exists as separate particles or short fibrils, the orientation of which is not appreciably affected by the stretching process. The stretching, however, does straighten out the cuticle which apparently improves the orientation

of the waxes. In the older fibers the longer, more firmly attached fibrils are pulled into better alignment by stretching. In the purified cellulose, the production of orientation by stretching is similar to that produced in rayons or other swollen cellulosic materials (13, 14).

Orientation in different varieties. Since the cotton samples were collected at various times during two seasons (1936 and 1937), a strict comparison regarding the age at which orientation developed in the three species is not possible, but the present data indicate no pronounced difference, with
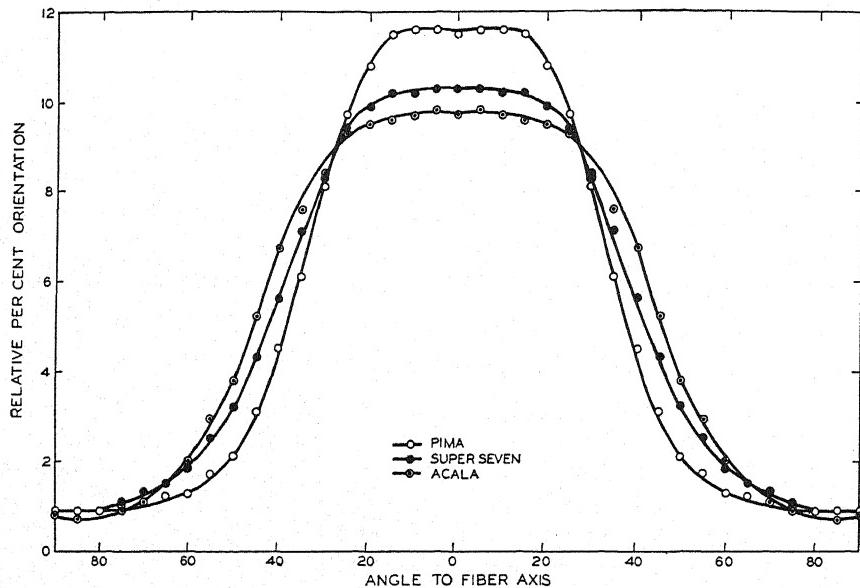
FIGURE 3. Comparative distribution curves showing the difference in preferred orientation of the three varieties of mature cotton, Pima, Super Seven, and Acala. [These curves were constructed as described in detail elsewhere (18).]

the exception that orientation may have developed slightly later in Pima. A quantitative comparison of the X-ray diagrams of the mature fibers, however, showed a detectable difference in the degree of preferred orientation, which is a maximum in Pima and minimum in Acala. Density distribution curves constructed (18) from the X-ray diagrams of the three varieties are shown in Figure 3. As explained in detail elsewhere (12), the shape of these distribution curves is due to the spiral orientation of the cotton fiber. These curves confirm the earlier qualitative observations of Clark, Pickett, and Farr (1) on the same varieties.

It is well known that the percentage of undeveloped or thin-walled fibers ("immaturity") has considerable influence upon the physical properties of raw cotton (10). For this reason it is of special interest to see if the

present data indicate a definite change in orientation with growth which might serve as a basis for estimating immaturity. Although no comparison was made with microscopic methods (10), the gradual change in orientation with age and the variation of orientation in different varieties appear to eliminate the possibility of a relationship which might serve as a basis for estimating immaturity. It is interesting, however, to note the possibility of a relationship between the variation of orientation in different varieties of mature cotton and the variation of orientation with tensile strength discussed elsewhere (15). Furthermore, since the decrease in preferred orientation (Pima > Super Seven > Acala) of mature fibers extends in the opposite direction from the decrease in number of fiber abnormalities (Pima < Super Seven < Acala) reported by Farr (3), a probable relationship is also suggested between these two factors.

SUMMARY

1. Cotton fibers of three varieties at various stages of growth were examined by X-ray diffraction methods to determine (a) the degree and type of orientation at various periods of cell wall development; (b) the orientation of noncellulosic constituents present; (c) the production of artificial orientation by stretching; and (d) the relative orientation of different varieties.

2. At each age the X-ray diagrams show a considerable degree of variation in orientation. In general, however, fibers younger than about 25 days give the X-ray pattern of randomly oriented cellulose; between approximately 25 and 35 days there develops a preferred orientation which does not change appreciably with further wall thickening. The type of orientation in all cases consists of a preferred orientation with reference to the fiber axis. There is no evidence of a selective orientation with reference to the surface of the cell wall.

3. The pattern of waxy materials, which is superimposed on that of cellulose in fibers younger than about 35 days, shows a preferred orientation in some of the samples.

4. Drying under tension improves the orientation of fibers showing a preferred orientation, while those showing a random orientation are not appreciably affected. Films and fibers prepared under tension from purified cellulose show a preferred orientation in all samples, regardless of the age of the fibers from which the cellulose was obtained.

5. In the mature fibers, the degree of orientation varies in the order Pima > Super Seven > Acala. The possible relationship of this to physical properties and fiber abnormalities is pointed out.

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LABORATORY COMPARISONS OF
COPPER FUNGICIDES[1]

S. E. A. McCallan and Frank Wilcoxon

Some thirty years ago Hedrick stated, "Twenty years of experimentation with copper compounds have given us none equal to Bordeaux mixture" (10, p. 146). After fifty years the interest and activity in this field is greater than ever and a number of new copper compounds have been developed (1, 12, 13, 23, 26, 27). In general the object has been to produce a copper compound possessing the desirable fungicidal and adhesive qualities of Bordeaux mixture but lacking its undesirable phytocidal properties, inconvenience of preparation, and conspicuous residue.

It is the purpose of this paper to present a laboratory evaluation of representative new copper preparations. The studies include detailed laboratory comparisons of toxic properties before and after exposure to a laboratory "rain" as well as limited phytocidal tests in the greenhouse and out-of-doors.

TOXICITY EXPERIMENTS

METHOD OF TESTING

The laboratory method of testing protective fungicides as developed and used in previous studies was employed (19, 20, 21). The copper compounds were placed in aqueous suspension and diluted to various appropriate concentrations. In general the dilutions varied by steps of one-half and one-fifth and the range from the greatest to least was 200-fold. The sprays were applied to clean glass slides by means of a No. 15 De Vilbiss atomizer. The atomizer was attached to the laboratory air pressure line and the pressure regulated to 40 mm. of mercury by means of a manometer. The duration of spraying was twelve seconds. At such a pressure and duration the spraying is completed just before the droplets start to coalesce on the slides.

After drying in the sun for three hours, or overnight, one series of the sprayed slides was subjected to the laboratory "rain." The "rain" consisted of distilled water delivered through a small rose-type nozzle. The perforations in the rose were 0.7 mm. in diameter. The slides were inclined on a rack and the rack oscillated beneath the falling "rain." The time of exposure was one minute and the pressure regulated to give from 0.5 to 0.6 inches of rainfall in this period (32). After this exposure the slides were allowed to dry for three hours and then placed in moist chambers to be compared with the slides which had not been subjected to rain.

The fungi selected for test organisms were: *Sclerotinia fructicola* (Wint.) Rhem., *Botrytis paeoniae* Oud., *Alternaria solani* (Ell. & Mart.) Jones & Grout, *Glomerella cingulata* (St.) Sp. & von. S., *Uromyces caryophyllinus* (Schr.) Wint., and *Gymnoconia peckiana* (Howe) Trotter. The first four fungi were grown on potato dextrose agar at room temperatures and the spores obtained by the vacuum technique (21). The spores of *S. fructicola* were from 5 to 7-day-old cultures, while the other three species were from 10 to 15-day-old cultures. The uredospores of *Uromyces caryophyllinus* were obtained from naturally infected greenhouse carnations (*Dianthus caryophyllus* L.) and the aeciospores of *Gymnoconia peckiana* from wild dewberries (*Rubus procumbens* Muhl.). All spores were suspended in distilled water at a concentration of approximately 45,000 to 65,000 spores

TABLE I

Copper Fungicides Studied in Comparison with Bordeaux Mixture

Fungicide	% Cu	Source
Oxo Bordeaux	13.0	Ansbacher-Siegle Corp., New York, N. Y.
Coposil	17.2	California Spray-Chemical Corp., Berkeley, Calif.
Copper compound KK (Copper oxychloride)	22.9	Röhm & Haas Co., Inc., Philadelphia, Pa.
Copper-Hydro "40"	26.8	Chipman Chemical Co., Inc., Bound Brook, N. J.
Z-O (Copper zeolite)	28.9	Nichols Copper Co., New York, N. Y.
Copper oxalate	39.5	R. O. Magie, N. Y. Agric. Exp. Sta., Geneva, N. Y.
Copper phosphate	45.4	M. C. Goldsworthy, U. S. Dept. Agric., Beltsville, Md.
Basic copper sulphate	52.0	The Mountain Copper Co., Ltd., San Francisco, Calif.
Cuprocide (Cuprous oxide)	88.2	Röhm & Haas Co., Inc., Philadelphia, Pa.

per cc. A duplicate series, exclusive of the rust spores, was run in a concentration of 0.02 per cent filtered orange juice (31). The spore suspensions were pipetted on to the sprayed slides, the slides placed in the customary moist chambers, and the percentage germination recorded after 24 hours. The tests were made at room temperature, 21° to 25° C.

Nine copper fungicides were tested and compared with Bordeaux mixture. The compounds are listed in Table I together with their copper content as determined by our analyses. The materials were obtained during the winter of 1936–1937. All comparisons were made on a basis of equal copper concentration. The Bordeaux mixture was prepared by adding a 2 per cent suspension of slaked calcium oxide to a 2 per cent solution of $CuSO_4 \cdot 5H_2O$. The resulting 4-4-50 mixture was diluted for the necessary comparisons.

METHOD OF EVALUATING RESULTS

The results of spore germination experiments are usually exhibited in the form of a toxicity curve, showing the percentage germination obtained at various concentrations of fungicide. In order to express the performance of a given fungicide by a single figure we may estimate the LD 50,

which is the concentration permitting 50 per cent of the spores to germinate.

In order to compare the LD 50 obtained from one fungicide with that obtained from another, it is necessary to attach to it a standard deviation, indicating the precision with which the LD 50 has been determined for a given lot of spores.

The exact treatment of such a problem has been developed by Bliss (3, 4, 5) in several recent papers. For the preliminary comparison of a number of fungicides, however, a somewhat simplified procedure may be used. It is necessary first to change the percentage germination values to other units tending to give a straight line toxicity graph instead of the usual S-shaped curve which is obtained when percentages are used. For this purpose Bliss has devised units called probits (3, 4) and has published tables for converting percentages to probits. Both percentages and probits, however, have the disadvantage that the observations plotted in these units do not all have equal weight, even when the same number of individuals has been counted in each observation. In a recent publication Bliss (5) has described a new unit, called the equivalent angle which is specially designed to overcome this difficulty. The relation between percentages and equivalent angles is given by the equation $p = \sin^2 \theta$, where p is the percentage and θ the equivalent angle. It is not necessary however to know this relationship since tables are furnished which make it a simple matter to change from percentages to equivalent angles. The effect of this change is to endow each observation with equal weight, assuming of course that the same number of individuals have been counted in each observation, and also to straighten partially the usual S-shaped toxicity curve.

The method of dealing with the original toxicity data was then as follows: The percentages were changed to equivalent angles and the latter plotted against the concentration of toxic agent. The best straight line passing through the mean angle and mean concentration was then drawn by eye, although this line may be fitted by the method of regression coefficients (28) if greater accuracy is desired. The LD 50 is estimated from the graph as the concentration at which the line crosses the 45° level (which corresponds to 50 per cent germination). It remains to estimate the standard deviation of the LD 50. A line may vary in two ways, in position and in slope, and all possible deviations of the LD 50 may be considered as being due to shifting of the line parallel to itself, or to a rotation about the mean, or to a combination of these. The variance of the LD 50 will then be the sum of two terms, one representing a parallel shift and the other a change in slope. The formula for the standard deviation (σ) is:

$$\sigma_{\text{LD50}} = \frac{1}{b} \sqrt{\frac{S(y-Y)^2}{n(n-2)} + \frac{S(y-Y)^2 \, (\text{LD } 50 - \bar{x})^2}{S(x-\bar{x})^2 \, (n-2)}}$$

The symbols have following significance:

b is the slope of the line.

$(y - Y)$ is the vertical distance of a point from the regression line.

$(x - \bar{x})$ is horizontal deviation of point from the mean of all the concentrations.

n is the number of points considered in drawing the regression line.

LD 50 $- \bar{x}$ is the difference between the LD 50 and the mean of the concentrations.

S signifies "sum of."

A typical example of this method of treatment is given in Table II. The original toxicity curve is plotted in Figure 1 A, and equivalent angles are plotted against concentration in Figure 1 B. A straight line passing through the mean equivalent angle and mean concentration has been fitted by eye to the central points, those at the extreme ends being excluded. The deviations from this line, read from the graph, to the nearest unit are shown in the last column of the table below. The deviations in concentration from the mean of concentrations are shown in the next to the last column. The LD 50, 0.0467 is read from the graph, and the standard deviation, 0.00346 is calculated as shown at the bottom of the table.

<div align="center">TABLE II</div>

EXAMPLE OF PROCEDURE FOR CALCULATING THE LD 50 AND ITS STANDARD DEVIATION

% germination	Equivalent angle (y)	Concentration (x)	$(x - \bar{x})^2$	$(y - Y)^2$
78.6	62.4	0.0086	0.000817	9
84.3	66.7	0.0086	0.000817	9
84.5	66.8	0.0086	0.000817	1
73.4	59.0	0.017	0.000408	0
58.8	50.1	0.017	0.000408	1
73.2	58.8	0.017	0.000408	81
28.2	32.1	0.086	0.002382	49
15.9	23.5	0.086	0.002382	1
17.4	24.7	0.086	0.002382	4
Totals	444.1	0.3348	0.010821	155
Means	49.34	0.0372		

$$b = -470, \ \text{LD } 50 = 0.0467, \ (\text{LD } 50 - \bar{x})^2 = 0.00009025$$

$$\sigma_{LD50} = \frac{1}{470} \sqrt{\frac{155}{63} + \frac{155 \times 0.00009025}{.01082 \times 7}} = 0.00346$$

<div align="center">RESULTS</div>

The results of the tests on the copper fungicides with the six fungi are shown in Figures 2, 3, and 4. In all cases the results for a given fungus were obtained from the same lot of spores and performed on the same day. The results shown in Figures 2 and 3 were obtained using orange juice, while
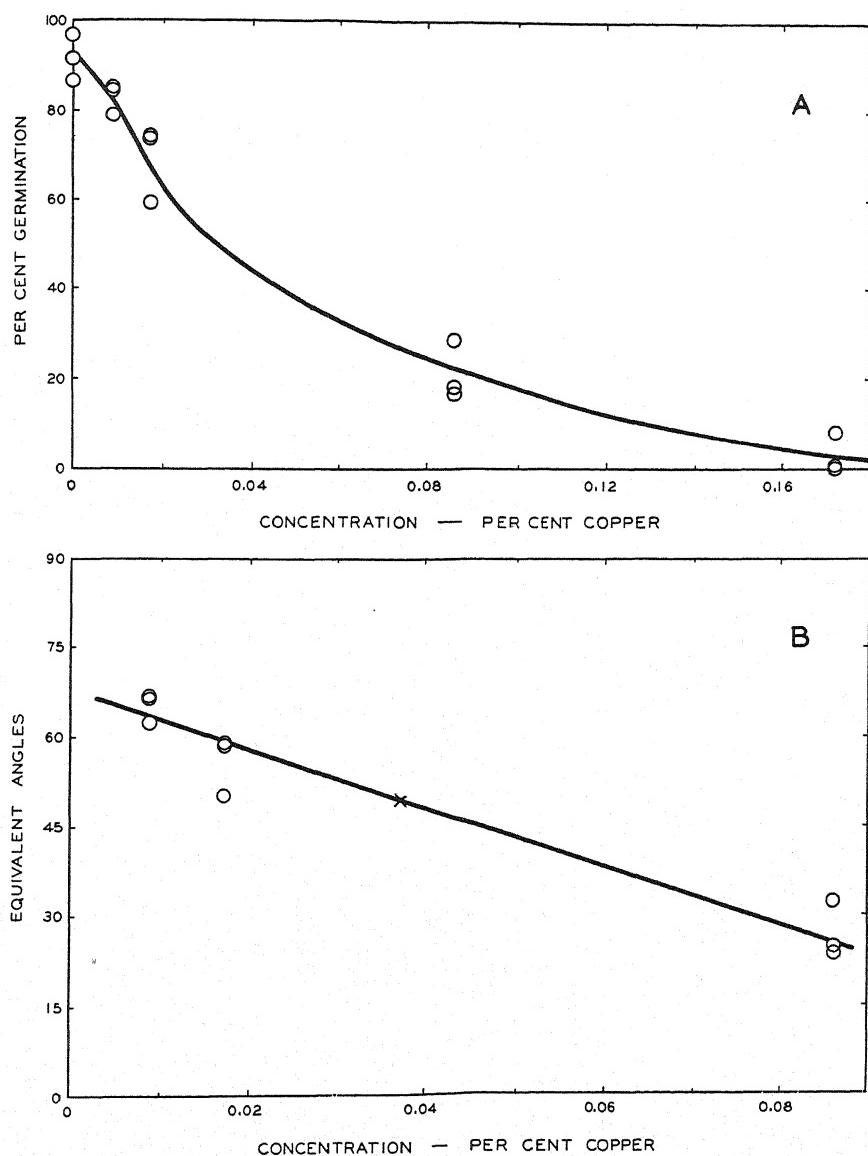
FIGURE 1. A. Toxicity curve with percentage germination plotted against concentration. B. Same data exclusive of extreme end points, with equivalent angles plotted against concentration
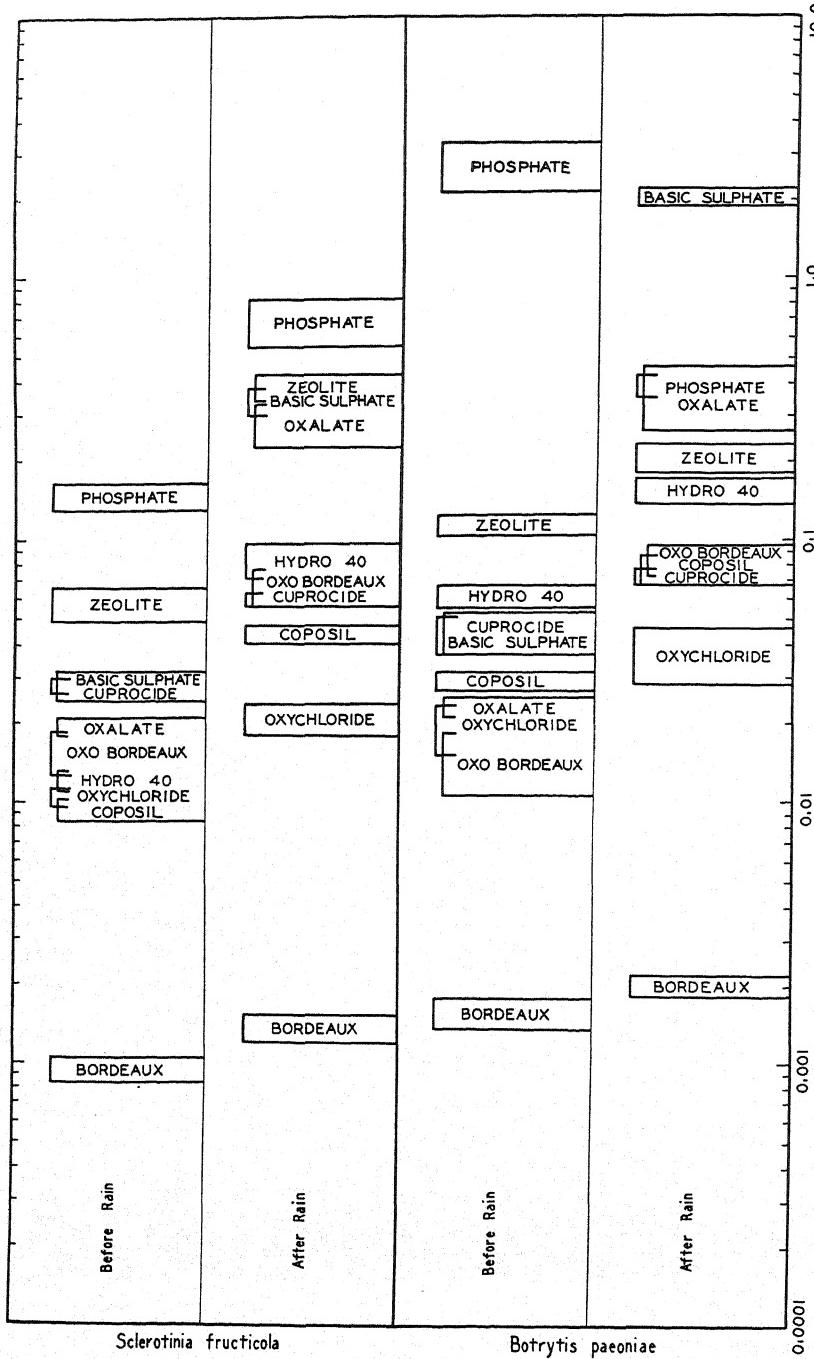
FIGURE 2. The *Sclerotinia fructicola* and *Botrytis paeoniae* LD 50 values for the copper fungicides plotted logarithmically. The width of each rectangle equals + and − the standard deviation of the LD 50.
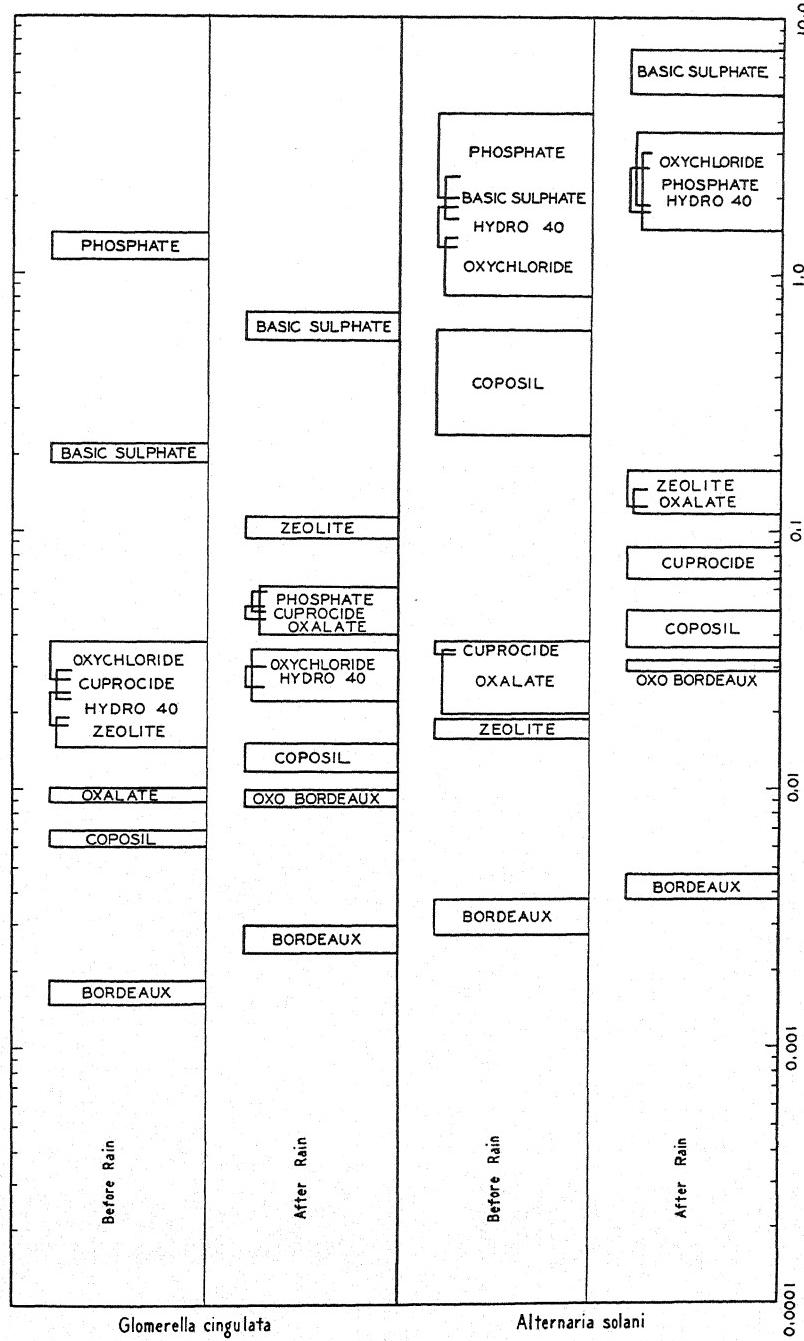
FIGURE 3. The *Glomerella cingulata* and *Alternaria solani* LD 50 values for the copper fungicides plotted logarithmically. The width of each rectangle equals + and − the standard deviation of the LD 50.
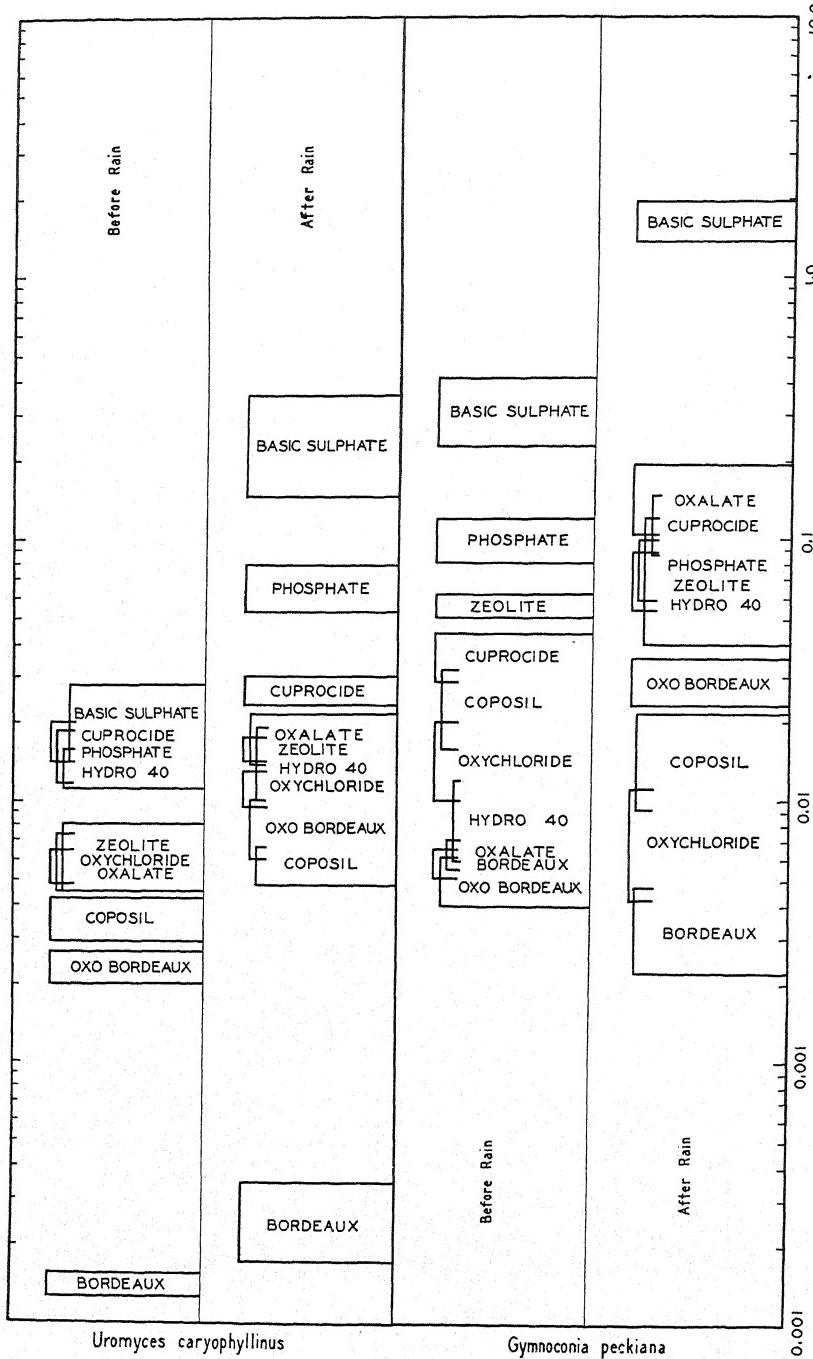
FIGURE 4. The LD 90 (*Uromyces caryophyllinus*) and LD 80 (*Gymnoconia peckiana*) values for the copper fungicides plotted logarithmically. Width of rectangles equals + and − standard deviation of the LD value.

in the case of Figure 4 no orange juice was used. The concentrations have been plotted logarithmically in order to show more readily the ratios between the LD 50 of the different compounds. Each compound is represented by a rectangle, the width of which is the range of + and − the standard deviation of the LD 50.

The difficulty of obtaining high percentages of germination with the rust spores necessitated employing respectively the LD 80 (20 per cent germination) and LD 90 (10 per cent germination) for the spores of *Gymnoconia peckiana* and *Uromyces caryophyllinus*. The variability and difficulty of obtaining satisfactory germination with the rust spores has been noted before (20).

The compounds, taking the toxicity results as a whole, may be grouped in three classes. Bordeaux mixture alone is in the first class, showing marked superiority to all other compounds. The third class consists of the copper phosphate and basic copper sulphate which are low in toxicity. The remaining compounds fall into an intermediate class and of these Oxo Bordeaux and Coposil are probably the most toxic and copper zeolite the least. It will be noted that in all cases except *Gymnoconia peckiana* Bordeaux mixture is from 4 to 20 times more toxic than the next best compound and 180 to 1800 times more toxic than the least effective preparation.

In general the compounds maintain relatively the same order of toxicity among the different fungi. An exception is copper oxychloride which has a high toxicity against *Sclerotinia fructicola* and *Botrytis paeoniae* but a much lower rating against the other fungi. Bordeaux mixture and to a lesser extent Cuprocide and copper oxychloride lose little of their inherent toxicity following exposure to rain. On the other hand, basic copper sulphate and copper oxalate decrease markedly in toxicity following rain tests. The results with copper phosphate are very erratic, in some cases its toxicity is apparently increased after rain.

TABLE III

RELATIVE ADHERENCE OF THE COPPER FUNGICIDES. PER CENT COPPER REMAINING ON GLASS SLIDES AFTER ONE MINUTE OF LABORATORY RAIN

Fungicide	Mean and standard deviation
Cuprocide	62.12 ± 6.21
Bordeaux	59.12 ± 7.35
Copper oxychloride	58.16 ± 1.85
Copper phosphate	40.68 ± 3.95
Coposil	27.88 ± 2.85
Copper-Hydro "40"	26.84 ± 2.00
Oxo Bordeaux	25.05 ± 11.40
Copper zeolite	10.66 ± 1.44
Copper oxalate	7.60 ± 2.18
Basic copper sulphate	3.68 ± 1.35

ADHERENCE TESTS

A chemical test of adherence, independent of the "after rain" toxicity test was made. Slides were sprayed with the different copper compounds at different concentrations, dried, and subjected to a one-minute laboratory rain test in the usual manner. An analysis of copper was then made on one set of slides before rain and another set after rain. These results are shown in Table III and substantiate the before and after rain toxicity tests. A similar order of adherence was obtained by Magie and Horsfall (22) on apple and cherry foliage.

PHYTOCIDAL OBSERVATIONS

Limited tests were performed in the greenhouse and out-of-doors to determine the relative injuriousness of the copper compounds to foliage. All comparisons were made on equal copper concentrations.

GREENHOUSE TESTS

Preliminary tests with 36 species of cultivated plants demonstrated that it is difficult to obtain copper injury under greenhouse conditions. A marked resistance was shown even to soluble copper sulphate. Four of the more sensitive species were, however, selected for comparative phytocidal tests. The plants were: lettuce (*Lactuca sativa* L. var. Big Boston), buckwheat (*Fagopyrum esculentum* Gaertn. var. Japanese), corn (*Zea mays* L. var. Golden Bantam), and bush bean (*Phaseolus vulgaris* L. var. *humilis* Alif. hort. var. Henderson). The plants were grown in four-inch pots until about 20 to 30 days old; they were then sprayed with the various copper compounds. Applications were made at concentrations of 0.4 and 2.0 per cent copper.

OUT-OF-DOORS TESTS

Injury tests on peach (*Prunus persica* Sieb. & Zucc.) and apple (*Pyrus malus* L.) were made out-of-doors during the month of May. The sprays were applied by hand atomizer to branches 18 to 24 inches long and bearing about the same number of leaves. All compounds were applied to an individual tree, care being taken to prevent the mixing of sprays by drifting. The applications at different concentrations were replicated on different trees. On the peach concentrations of 0.02 and 0.1 per cent copper were used and on the apple 0.1 and 0.5 per cent.

RESULTS

The results of the sprays on the greenhouse plant were characterized by the extent of injury. Out-of-doors definite necrotic spots appeared on the leaves of apple and peach and it was possible to classify the leaves into groups depending on the varying degrees of injury.

The general results are presented in Table IV where the copper compounds are placed in groups based on their effect on the different plants. Phytocidal differences between compounds within a group could not be demonstrated.

It will be seen that in general the more fungicidal compounds are also the more phytocidal. However, Bordeaux mixture is not outstanding in

TABLE IV

Relative Phytocidal Responses of Different Plants to the Different Copper Fungicides

Injury	Peach	Apple	Lettuce	Buckwheat	Corn	Bush bean
Very severe	Coposil Cuprocide Oxychloride					
Severe	Oxo Bordeaux Basic sulphate Bordeaux Phosphate	Cuprocide Bordeaux Coposil Oxychloride Basic sulphate				
Moderate	Zeolite Hydro "40" Oxalate	Zeolite Oxo Bordeaux Hydro "40"	Bordeaux Oxo Bordeaux Coposil Cuprocide	Coposil Cuprocide		
Slight		Phosphate Oxalate	All others	All others	Coposil	Coposil
None					All others	All others

foliage injuriousness as it is in toxicity to fungous spores. Coposil, Cuprocide, copper oxychloride, Oxo Bordeaux, and basic copper sulphate all equal or exceed Bordeaux in phytocidal action, while copper zeolite, Copper-Hydro "40," copper phosphate, and copper oxalate produce less injury.

DISCUSSION

The outstanding result of these comparisons is the marked superiority of Bordeaux mixture in toxicity under all conditions. This superiority is equally pronounced before rain and so cannot be ascribed to the greater adherence of Bordeaux. Nor can it be due to the lime content as toxicity tests with lime have shown little or no toxicity at concentrations comparable to that of the Bordeaux mixture dilutions. Since the Bordeaux precipitate has a gelatinous character quite different from most of the proposed substitutes, it is possible that its superior toxicity is due to its physical nature. However, further work will be necessary to explain this superiority.

The details of few laboratory comparisons of these copper preparations

are available. However, Nikitin (25) found Bordeaux more toxic than copper zeolite, and copper zeolite more toxic than copper phosphate, copper hydroxide, or basic copper sulphate. The test spores were from *Macrosporium solani* and *Glomerella cingulata*. Horsfall, Marsh, and Martin (15) prepared a number of cuprous and cupric oxides and concluded that cuprous oxide was more toxic than the cupric. Bordeaux mixture was shown to be more toxic than various basic copper sulphates in the laboratory tests of Holland, Dunbar, and Gilligan (11). No investigators appear to have found an "insoluble" copper compound inherently more toxic to funguous spores than Bordeaux mixture.

A good review of the recent developments in new copper fungicides is available in a paper by Marsh (23). It is apparent that the results of field experiments have not been consistent and no compounds have been outstandingly good even on one crop. For example in potato spraying trials Marsh, Martin, and Munson (24) found Bordeaux mixture and cuprous oxide most effective in one season and cupric oxide more effective in two other seasons. Tilford's results (29, 30) with potatoes showed copper oxychloride the only compound equal to Bordeaux one season, while the next season Bordeaux was superior to copper oxychloride. Coposil and Copper-Hydro "40" sprayed potatoes out-yielded Bordeaux in Bonde's test (6). Wilson (33) and Wilson and Runnels (34) consider copper oxychloride the most promising substitute for Bordeaux on vegetable crops. These investigators found that Bordeaux gave the best control of *Alternaria* blight on ginseng. Horsfall and Hamilton (13) obtained good control of several diseases with Cuprocide. Adams and Nikitin (1) report copper zeolite a more desirable spray on apple than Coposil, Cuprocide, basic copper sulphate, or Bordeaux. In the control of apple blotch Kadow and Anderson (18) found a number of different compounds satisfactory. The results of Cation and Rasmussen (7, 8) indicate that a number of the copper compounds will control cherry leaf spot, while Anderson and Kadow (2) found only a copper oxychloride and a modified Bordeaux satisfactory. Roberts *et al.* (26) have reported promising results with copper phosphate, as also have Sessions (27) and Hamilton (9) with Coposil. Young and Beckenbach (37) have pointed out that many of the new compounds have limited value as fungicides due largely to a lack of adhesiveness.

Various investigators (14, 33, 35, 36) have shown that certain crops such as tomatoes and cucurbits, while normally copper-resistant, are subject to Bordeaux injury. This injury is apparently due to the lime (16, 35). Under these circumstances a suitable Bordeaux substitute would be very desirable. Cuprocide has given satisfactory results on tomatoes and greenhouse tomato seedlings in the experiments of Horsfall, Magie, and Cunningham (14) and Horsfall and Suit (17), while Wilson and Runnels

(35, 36) recommend copper oxychloride as a substitute for Bordeaux on tomatoes, and copper oxychloride or Cuprocide for tomato transplants.

The erratic results of field experiments and their failure to demonstrate any outstanding compounds is probably due to the large number of variables involved in field tests, the preliminary nature of many of the reports, the failure in many cases to base comparisons on equal copper contents, and the fact that many of the compounds probably do not differ significantly.

SUMMARY

1. A laboratory comparison was made of Bordeaux mixture and nine other representative copper compounds with respect to toxicity, adherence, and foliage injury. These evaluations were based on equal copper concentrations.

2. Details are given for a method of calculating the LD 50 and its standard deviation from the toxicity curves of compounds tested in the laboratory.

3. The results of laboratory toxicity determinations both before and after exposure to laboratory "rain" with the spores of *Sclerotinia fructicola, Botrytis paeoniae, Glomerella cingulata, Alternaria solani, Uromyces caryophyllinus*, and *Gymnoconia peckiana* demonstrate the marked superiority of Bordeaux mixture over all other compounds. Copper phosphate and basic copper sulphate are very inferior in toxicity. An intermediate class contains Oxo Bordeaux, Coposil, copper oxychloride, Cuprocide, Copper-Hydro "40," copper oxalate, and copper zeolite. Oxo Bordeaux and Coposil are probably the most toxic of this class and copper zeolite the least.

4. Adherence tests based on chemical analyses and toxicity before and after laboratory "rain" show Bordeaux mixture, Cuprocide, and copper oxychloride to be the most adherent and copper oxalate and basic copper sulphate the least.

5. Limited phytocidal tests in the greenhouse on lettuce, buckwheat, corn, and bush bean, and out-of-doors on peach and apple indicate that in general those compounds most toxic to fungous spores are also most toxic to the leaves of green plants. The difference in phytocidal properties is not, however, as great as that of fungicidal properties and all compounds out-of-doors injure to some extent. Compounds equal to or exceeding Bordeaux in injury are: Coposil, Cuprocide, copper oxychloride, Oxo Bordeaux, and basic copper sulphate, while compounds producing less injury than Bordeaux are: copper zeolite, Copper-Hydro "40," copper phosphate, and copper oxalate.

6. No copper compound appears to have been developed which combines high fungicidal value with low phytocidal properties. Bordeaux mixture has at least as great a range in this respect as any other compound.

In special cases such as lime-sensitive plants certain of these compounds may be more desirable than Bordeaux.

7. These results indicate that fifty years of experimentation with copper compounds have given us none equal to Bordeaux mixture.

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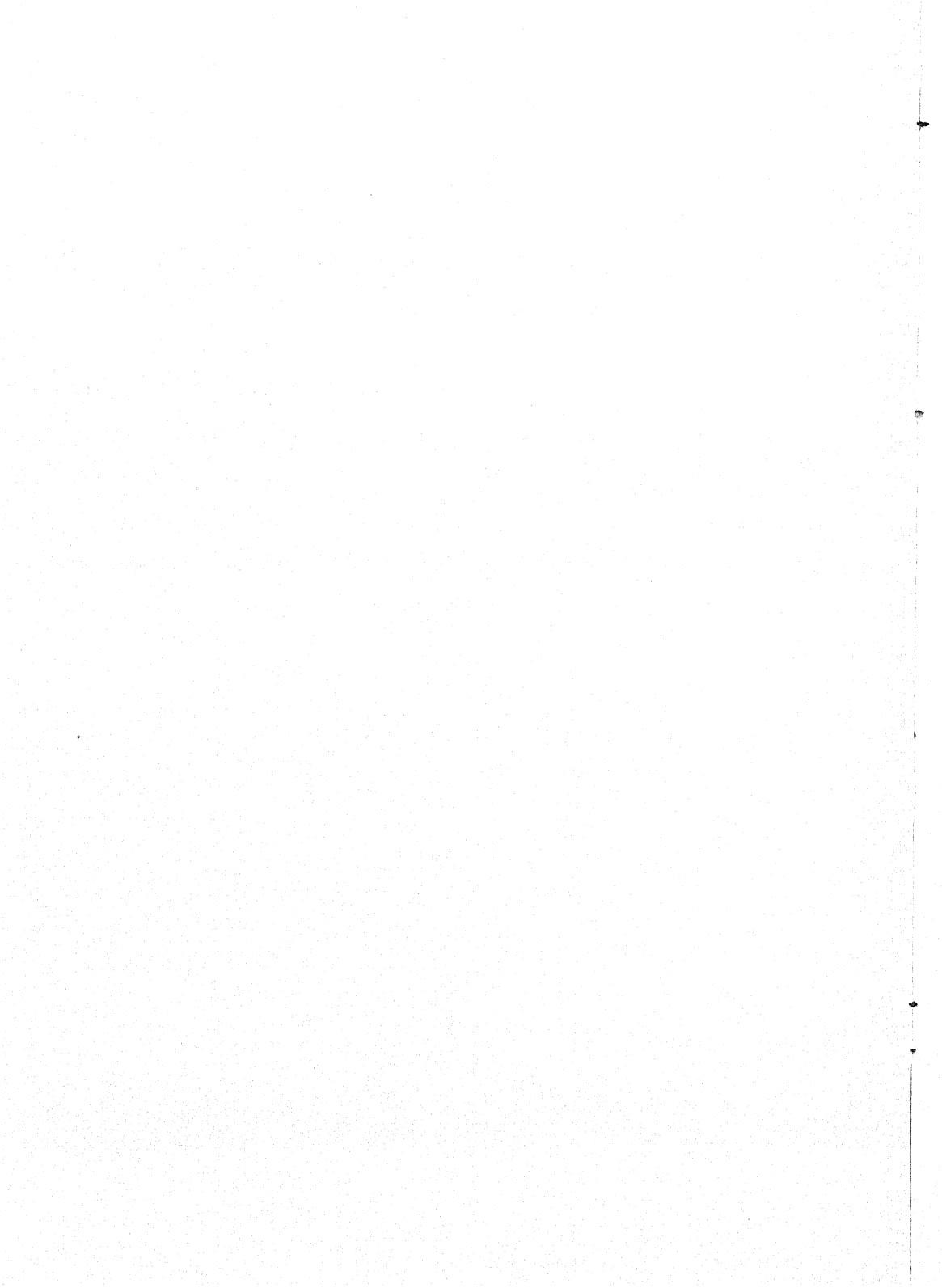
EFFECT OF ETHYLENE THIOCYANOHYDRIN, ETHYL CARBYLAMINE, AND INDOLEACETIC ACID ON THE SPROUTING OF POTATO TUBERS[1]

John D. Guthrie

During the course of the investigation of the effect of stimulative chemicals on plants, observations have been made from time to time which have not warranted separate publication. Two of these, the effect of ethylene thiocyanohydrin and of ethyl carbylamine, have been combined in the present paper. To this a preliminary report on the recently observed inhibiting effect of neutralized indoleacetic acid on the sprouting of nondormant potato tubers has been added. It has also been noted that potassium indoleacetate induced roots to grow from the cut surface of potato tubers.

EFFECT OF ETHYLENE THIOCYANOHYDRIN

Since both ethylene chlorohydrin and potassium thiocyanate have been shown by Denny (1) to be very effective in breaking the dormancy of potato tubers, it seemed of interest to try a compound that was similar chemically to both of these, that is ethylene thiocyanohydrin, $CH_2SCN \cdot CH_2OH$. Although no reference to the preparation of this compound could be found, a procedure for preparing a substance that appears to be $CH_2SCN \cdot CH_2OH$ has been worked out.

Ethylene bromohydrin, 50 g., was refluxed with 39 g. KSCN and 300 cc. of absolute alcohol for 3 hours. The KBr, 42 g., formed in the reaction was filtered out, the alcohol evaporated *in vacuo* in a bath at 50° C., and the residue taken up in 450 cc. of ether. After standing for several hours at −10° C., 4 g. of unused KSCN was filtered out, the solution dried with Na_2SO_4 and evaporated *in vacuo* to 150 cc. The solution was placed in a separatory funnel and about 50 cc. of petroleum ether added to bring down about 3 cc. of a reddish oil which was discarded. The solution was evaporated *in vacuo* until most of the solvent was removed and the residue washed twice with 50 cc. petroleum ether. The solvents were then completely removed from the liquid *in vacuo* at 60° C. The yield was 32 g. of a clear oily liquid. Its sulphur content was 28.2 per cent. This sample was used for treatment of potato tubers. Another sample was further purified by fractional precipitation from ether with petroleum ether. It contained 30.4 per cent sulphur and had a specific gravity of 1.223 at 25° C. The theoretical sulphur content for $CH_2SCN \cdot CH_2OH$ is 31.1 per cent. Although the product is believed to be ethylene thiocyanohydrin, the possibility that it is an isomer of this compound has not been eliminated.

The potatoes (*Solanum tuberosum* L.) used were second crop Irish Cobbler tubers, harvested in New Jersey in late October, 1934. Pieces cut with one eye were soaked for one hour in solutions of ethylene thiocyanohydrin and then planted without rinsing. The control was soaked in water, and for comparison treatments were also made with potassium thiocyanate. A record was kept of the appearance of sprouts above ground. For this two flats of twelve pieces each were used. The results are shown in Table I where the time for 50 per cent and 90 per cent of the pieces to show sprouts above ground is reported. It will be seen that ethylene thiocyanohydrin is very effective in the first experiment at 2.4 g. per liter, while in the second experiment on the same tubers 10 days later, it was effective at 1 g. per liter. In both experiments higher concentrations were tried, but some of the pieces rotted. It may be concluded that ethylene thiocyanohydrin at 1 to 2 g. per liter is almost as effective as potassium thiocyanate at 10 g. per liter in producing early sprouting as measured by the time for 50 per cent above ground, but is distinctly less effective in producing uniform sprouting as measured by the time for 90 per cent above ground.

EFFECT OF ETHYL CARBYLAMINE

Since a sample of ethyl carbylamine (C_2H_5NC) which had been prepared for another purpose was available, it was tried on dormant potato tubers. The ethyl carbylamine was prepared from 100 g. silver cyanide and 60 cc. of C_2H_5I by the method of Gautier (4, p. 233) with minor modifications and purified by the method of Toda (12). A fraction boiling at

TABLE I

EFFECT OF ETHYLENE THIOCYANOHYDRIN AND ETHYL CARBYLAMINE ON THE
SPROUTING OF DORMANT POTATO TUBERS

Treatments		Days for 50 per cent above ground	Days for 90 per cent above ground
Ethylene thiocyanohydrin	2.4 g. per l.	38	58
" "	1.2 g. " "	67	75
Control—H_2O		95	>107
Potassium thiocyanate	10 g. per l.	24	31
" "	5 g. " "	54	82
" "	2 g. " "	74	89
Ethylene thiocyanohydrin	1 g. per l.	32	48
" "	0.5 g. " "	57	78
Control—H_2O		69	81
Potassium thiocyanate	10 g. per l.	28	31
" "	5 g. " "	55	74
" "	2 g. " "	62	70
Ethyl carbylamine	0.75 g. per kg.	21	28
" "	0.25 g. " "	23	38
" "	0.08 g. " "	60	70
Control—closed container only		60	70

77° to 78° C. was used for the treatments. The tubers used were from the same source as described above but were of the 1937 crop. Treatments were made on whole tubers by the vapor method of Denny (1). One kg. of tubers was placed in 3.5 liter containers for 24 hours with the amounts
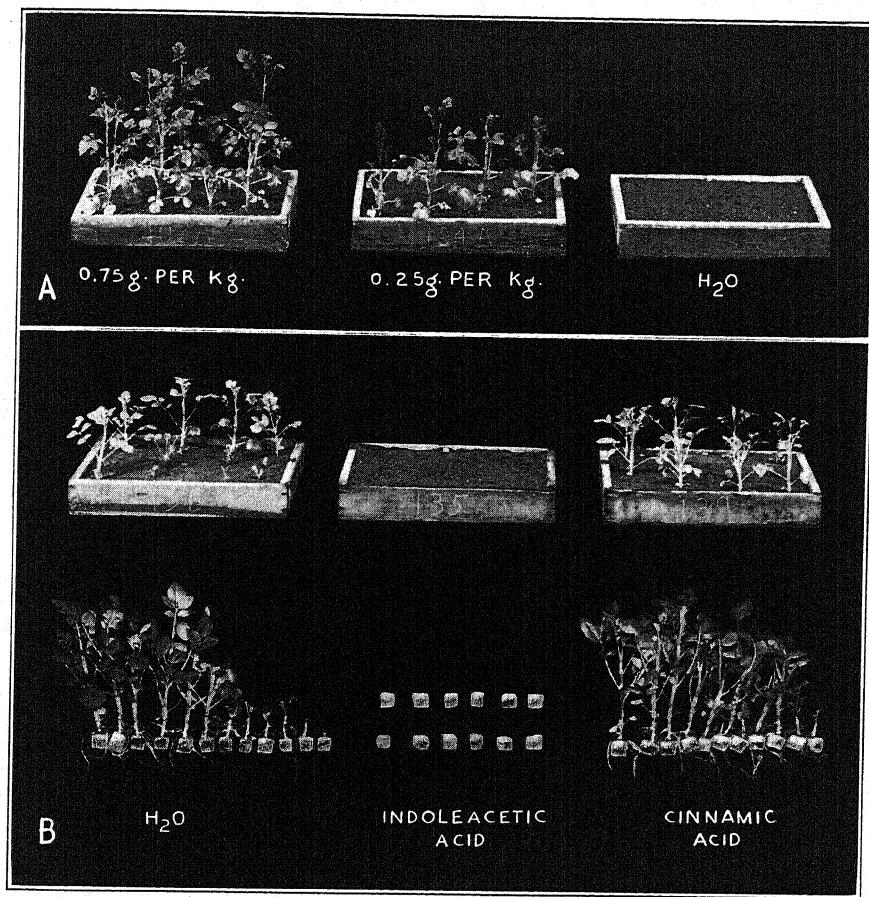
FIGURE 1. (A) Effect of ethyl carbylamine on the sprouting of dormant potato tubers; (B) inhibiting effect of indoleacetic acid on the sprouting of non-dormant potato tubers, with pieces treated with water and with cinnamic acid as controls. Lower row same plants as in upper row, but dug up to show that treated pieces have not rotted.

of ethyl carbylamine given in the table and then cut into one-eye pieces and planted. The sprouting data was taken from 24 pieces of each lot. The results given in Table I and in Figure 1A show that ethyl carbylamine is very effective in breaking the dormancy of potato tubers. However, it is doubtful if it could be used in a practical way on account of its objectionable odor and poisonous nature.

EFFECT OF NEUTRALIZED INDOLEACETIC ACID ON
THE SPROUTING AND ROOTING OF NON-
DORMANT POTATO TUBERS

Following the papers of Thimann and Skoog (9, 11) which showed that
application of indoleacetic acid and other auxins inhibited the growth of
lateral buds of decapitated seedlings of *Vicia faba* and of peas, experi-
ments were started to see if indoleacetic acid would inhibit the growth of
the buds of non-dormant potato tubers. It is only recently that it has been
possible to obtain a marked inhibiting effect. In earlier experiments,
various methods of applying indoleacetic acid were tried. It was applied
by soaking cut pieces in solutions ranging from 10 to 200 mg. per liter
for 1 to 3 hours, by application in lanolin near the eyes in concentrations
up to 50 mg. per gram, by spraying the tubers with a solution of 100 mg. in
5 cc. olive oil, and by means of drawn out glass tubes in concentrations of
300 mg. per liter of water. In some cases no inhibiting effect on sprouting
was observed and in none of the experiments was the sprouting retarded
more than one week.

In some experiments reported recently (5) on the effect of glutathione
on the sprouting of dormant potato tubers it was found that this substance
entered the tissue with difficulty, but that sufficient glutathione could be
gotten into the tissue to have a dormancy breaking action by placing
pieces with their cut bases in 2 per cent solutions for 3 days at 10° C. Re-
cently it has been found that such treatments may be continued for 6
days with better results. This procedure was tried with indoleacetic acid,
since it seemed likely that sufficient of this substance to have an inhibiting
effect could be introduced into the tissue by this means.

Inhibiting effect on sprouting. In experiment 1 of this new series which
was started January 25, 1938, Irish Cobbler tubers harvested in New
Jersey in November were used. These were just coming out of their rest
period. Cut pieces having one eye and weighing about 10 g. were used.
They were washed well with water, dried, and placed in a petri dish with
the eye up. A solution of 100 mg. of indoleacetic acid neutralized with
5.7 cc. N/10 NaOH and made up to 100 cc. was poured into the dish. After
standing uncovered for 3 days at 10° C., a thin layer was cut from the bot-
tom of the pieces, the pieces washed and a new solution applied for 3 more
days. The pieces were then planted in the greenhouse at about 20° C.
Twelve pieces were used for the treatments and two controls of 12 pieces
each were made in the same way using water. When observed February 11,
the pieces in the control sample had large sprouts, but there was no sign
of sprouting in the treated lot. One-half of the pieces had put sprouts above
ground on February 15 in one control, and on February 17 in the other,
an average of 22 days for 50 per cent above ground from the start of the
experiment and 16 days from the time of planting. The treated pieces,

however, showed no signs of sprouting. When observed 40 days from the start of the experiment there were no signs of sprouting in the treated lot although the pieces were in sound condition. Eleven sprouts had appeared in each control. In this experiment a treatment with 100 mg. of neutralized cinnamic acid per 100 cc. showed 50 per cent above ground in 21 days from the start of the experiment. The photographs used for Figure 1B were taken March 7.

TABLE II

Inhibiting Effect of Neutralized Indoleacetic Acid and Other Substances on the Sprouting of Non-Dormant Potato Tubers

Exp.	Treatment	No. sprouts above ground				
		Feb.		March		
		24	28	3	7	15
2	100 mg. indoleacetic acid per 100 cc.	0	0	0	0	0
	25 mg. " " " " "	0	0	0	0	3
	10 mg. " " " " "	0	0	0	7	12
	H₂O	0	0	2	11	11
	300 mg. cinnamic acid per 100 cc.	0	0	3	7	8
	H₂O	0	1	5	10	12
3	125 mg. pot. indoleacetate per 100 cc.	0	0	0	0	1
	30 mg. " " " " "	0	0	0	2	5
	12 mg. " " " " "	0	2	9	12	12
	H₂O	0	10	12	12	12
	125 mg. pot. α-naphthylacetate per 100 cc.	0	0	0	0	0
	H₂O	0	1	8	12	12
4	Planted at once	4	9	12	12	12
	H₂O 1 day	1	8	11	12	12
	H₂O 3 days	1	9	11	12	12
	H₂O 7 days	0	0	8	11	12
	Pot. indoleacetate 1 day	0	0	0	0	6
	" " 2 days	0	0	0	0	3
	" " 3 days	0	0	0	0	0
	" " 4 days	0	0	0	0	1
	" " 7 days	0	0	0	0	2

Other experiments were started as soon as this strong inhibiting effect was noted. The results are shown in Table II. All the treatments were made by the basal soak method at 10° C. as described above. In experiment 2 started February 11, the same lot of tubers was used as in experiment 1 and 10 g. pieces were used. In experiment 3 started at the same time another lot of non-dormant Irish Cobblers was used. Some of the eyes on these tubers showed small sprouts, but 10 g. pieces were chosen with non-sprouting eyes and well mixed before distribution to the various lots. In experiment 4 started February 14, the same tubers were used as in experiment 3, but the pieces weighed about 20 g. The solutions used for this experiment were 150 mg. of potassium indoleacetate per 125 cc. water. Solutions were changed at 3 days in the 4 and 7 day treatments as described in the first experiment.
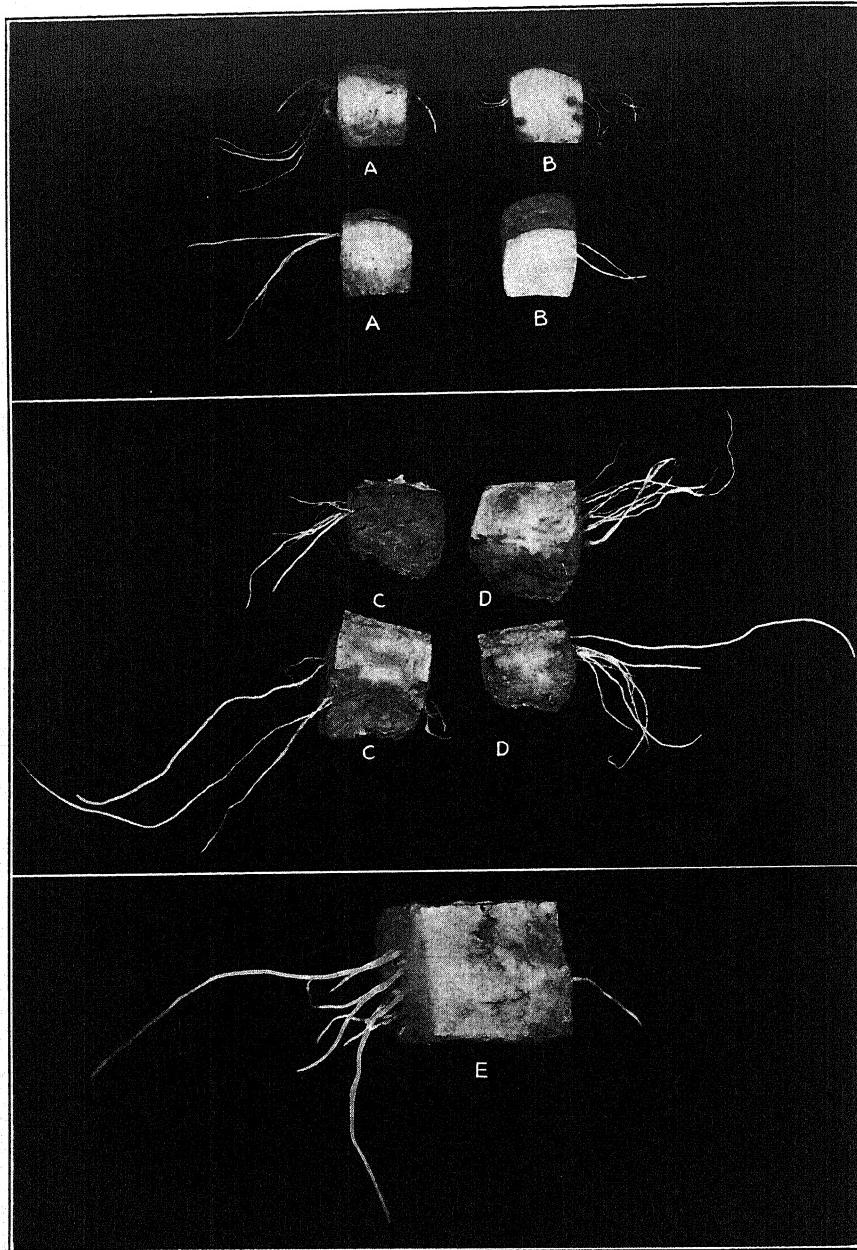
FIGURE 2. Rooting response of pieces of potato tubers treated with indoleacetic acid. (A) Pieces from exp. 2, 100 mg. per 100 cc. (B) Pieces from exp. 2, 25 mg. per 100 cc. (C) Pieces from exp. 4, potassium indoleacetate 150 mg. per 125 cc. for 1 day. (D) Pieces from exp. 4, potassium indoleacetate 150 mg. per 125 cc. for 2 days. (E) Piece from exp. 2, 100 mg. per 100 cc., natural size.

Table II shows plainly that treatments with neutralized indoleacetic acid inhibits the sprouting of the tubers. The treated pieces were in good condition with the exception of the potassium naphthylacetate treatment of experiment 3, in which half of the pieces rotted. Weaker treatments of this substance will be tried. The proliferation at the lenticels and the appearance of small sprouts in some of the weaker treatments showed that the treated pieces were alive. Although some pieces in the stronger treatments showed surface rot, other chemicals have been noted to produce surface injury without inhibiting sprouting. It may be noted, however, that inhibition of potato sprouting by thioacetamide has been noted by Miller (8) and by ethylene by Elmer (2), Gane (3), and Huelin (7).

The question as to whether tubers that are naturally dormant contain more auxin than non-dormant tubers must be answered analytically before a connection between the above noted inhibition and dormancy can be shown. Several years ago this was undertaken, but methods of extraction then available appeared to be inadequate. Using Thimann's chloroform, hydrochloric acid procedure (10) no substance active on oat coleoptiles could be obtained from freshly-harvested potato tubers, but there were indications that the tubers contained something that destroyed auxin in the process of extraction. Active extracts were obtained from potato sprouts. From time to time as improved methods of extraction become available, this phase of the investigation will be continued. It is also planned to treat some of the inhibited pieces with dormancy-breaking chemicals to see if they will hasten the sprouting of such pieces.

Rooting response. The question of whether or not the pieces were prevented from growing due to injury is best answered by the growth of roots from the cut surface of many of the treated pieces. These roots grow from the cut surface well away from the eye or skin. This is illustrated in Figure 2. Roots do not normally grow from the cut surface. Such rooting has not been seen on potato tubers before in this Laboratory, although thousands of pieces have been observed. It is impossible to state at the present time that rooting from the cut surface of potato tubers has never been reported before, but if this were true, the case is a good one for the induction of roots in a place where they never appear without treatment with a root-inducing substance like those used so successfully by Hitchcock and Zimmerman (6, 13).

SUMMARY

1. A substance believed to be ethylene thiocyanohydrin was effective in breaking the dormancy of potato tubers. Ethyl carbylamine also showed marked dormancy-breaking action.

2. Neutralized indoleacetic acid inhibited the sprouting of pieces of non-dormant potato tubers when the bases of the pieces were soaked in solutions ranging from 25 to 100 mg. per 100 cc. for 1 to 7 days at 10° C.

3. These treatments with neutralized indoleacetic acid also induced rooting at the cut surface of the pieces. Rooting at this part of potato tubers has not been observed before in this Laboratory, nor have we found a report of such rooting in the literature.

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EXTRACTION AND DETERMINATION OF VITAMIN C IN PLANT TISSUE

Norwood C. Thornton

The usual procedure of extraction recommended by Bessey and King (1) requires considerable time for separating the vitamin C from the tissue after the tissue has been macerated. Because of this it was found necessary to resort to some other procedure when as many as 25 samples of tissue had to be examined within a few hours. During the past year the procedure as previously outlined (3) and as herein presented has been used with complete success. The data presented in the tables in this report have been obtained exclusively with the banana (*Musa sapientum* L.) fruit.[1] However, the efficiency of the procedure has been checked by measurements with potato (*Solanum tuberosum* L.) tubers; asparagus (*Asparagus officinalis* L.) shoots; apple (*Pyrus malus* L.) fruit; bean (*Phaseolus vulgaris* L.); pea (*Pisum sativum* L.); and tangerine (*Citrus nobilis* L.).

METHOD AND RESULTS

Procedure recommended. The plant tissue was rapidly sampled, weighed (approximately 20 grams), and placed in a porcelain mortar containing 50 cc. of a mixture of acids. This acid mixture consisted of equal parts of either 1 N, 2 N, or 3 N sulphuric acid and N/4 metaphosphoric acid as suggested by Mack and Tressler (2). The tissue was ground with a porcelain pestle by the aid of 3 to 8 grams of acid-washed quartz sand to an extremely fine consistency. This preparation was then transferred to a 200 cc. volumetric flask with repeated rinsings of the mortar and made up to volume with the acid mixture, thoroughly mixed, centrifuged, and aliquots used for titration with a solution of 2.6-dichlorophenolindophenol. The indicator solution was prepared each day and was standardized against a freshly prepared solution of ascorbic acid "Roche" in the acid mixture used on the particular plant tissue tested. Sufficient indicator may be prepared for two or three days' use, but this solution must be standardized against a freshly prepared ascorbic acid solution each time it is used. Since the indicator slowly loses in strength and a reddish-brown coloration appears which interferes with the end point upon standardization it is recommended that the solution should not be used for more than two days. The dye (approximately 0.1 to 0.2 g. per liter) is dissolved in a small quantity of distilled water, filtered and diluted to a point where the meniscus is easily determined in the micro-burette. Since carbon dioxide

[1] The writer is indebted to Mr. G. L. Poland of the United Fruit Company for furnishing the bananas used in this work.

causes a reduction in the strength of the dye solution it is necessary to dissolve the dye in distilled water at room temperature which is in equilibrium with the carbon dioxide of the atmosphere in order to overcome any great change in the dye, from this source, after it has been standardized.

The titration of the plant extract with the dye is facilitated by the following procedure: The micro-burette is set up with two large test tubes or centrifuge tubes. A 10 cc. aliquot of the extract is measured into each of the tubes. The titration is carried out by running the dye solution from the burette into the sample which is constantly agitated by tapping the lower end of the loosely clamped tube with the finger. The end point of the titration is readily determined by comparison with the adjacent tube containing only the extract. This is particularly useful when titrating extracts containing some coloring matter. With banana tissue extract the end point is reached at the point where the pink color of the extract persists for approximately 10 seconds with constant agitation of the solution.

It is true that the acid solution used for extracting vitamin C from the tissue will discolor the dye, but this is of little practical consequence. During this investigation all of the various combinations of acid extracting media used as well as an acid mixture wash of the quartz sand were titrated with the dye solution with the result that less than one drop (or 0.04 cc.) of dye was necessary to develop a pink color which is an amount too small to interfere with the determination of vitamin C.

Treatment of the tissue extract with hydrogen sulphide (2) is often necessary to insure that all of the ascorbic acid present is in the reduced form which is the only form that will react with the dye. This procedure is usually unnecessary when a strong acid is employed to extract the ascorbic acid from the tissue. However, when an unknown tissue is being investigated or there is any doubt of the efficiency of the acid mixture in inactivating the enzymes, the hydrogen sulphide treatment of the tissue extract should be employed.

This procedure is carried out most easily by taking an arbitrary volume of extract in a graduate and passing H_2S through the liquid for 10 to 15 minutes and letting the solution stand for an additional period up to 15 minutes, then bubbling carbon dioxide through the solution until all of the H_2S is removed. The solution is then made up to the original volume with the acid mixture if there has been any loss by evaporation. Aliquots are then taken for titration with the dye.

This volumetric procedure has been used with various concentrations of different acids often recommended and used for the determination of vitamin C in plant tissue. The results of these tests are given in Table I where the data corresponding to the banana finger used are analyses of from two to five samples taken from the same finger. Of the three acids tested in combination with metaphosphoric acid only sulphuric gave con-

TABLE I

VITAMIN C CONTENT OF BANANAS AS DETERMINED BY VOLUMETRIC PROCEDURE
WITH VARIOUS ACID MEDIA

Color and condition of banana (finger)	H₂S reduction of extract before titration	Vitamin C, mg. per 100 g. of fresh tissue						
		Acid medium used with metaphosphoric acid						
		Sulphuric			Acetic			Trichloracetic
		1 N	2 N	3 N	18%	8% cold	8% hot	8%
Yellow, firm	No	12.8	—	—	10.3	8.6	—	12.8
	Yes	12.8	—	—	11.0	10.1	—	12.8
Yellow, firm	No	12.9	—	—	10.1	8.2	—	12.7
	Yes	12.9	—	—	10.9	9.0	—	12.7
Green*	No	11.4	—	—	8.9	6.9	8.1	10.4
	Yes	11.4	—	—	10.0	8.1	9.3	10.6
Green**	No	13.1	—	—	12.2	7.7	7.4	—
	Yes	13.1	—	—	12.9	7.9	9.0	—
Green***	No	15.0	15.0	—	—	—	—	14.0
	Yes	15.0	15.0	—	—	—	—	14.3
Yellow, firm	No	13.0	12.9	13.0	—	—	—	—
Yellow, firm	No	12.6	12.6	12.5	—	—	—	—
Green***	No	15.0	15.1	15.1	—	—	—	—
	Yes	15.0	15.1	15.1	—	—	—	—

* Exposed to respired CO₂ for 48 hours.
** Held in air 24 hours after exposure to CO₂.
*** Normal condition.

sistent and uniformly high values which were unchanged upon reduction with hydrogen sulphide. Sulphuric acid may be used in any one of three concentrations, i.e., 1 N, 2 N, or 3 N. The strong acid has no detrimental effect on the ascorbic acid content of the plant tissue as shown by the data given under these three concentrations. Furthermore, it is not essential that the sulphuric acid extract be titrated immediately. Tissue extracts were held in stoppered flasks at room temperature for as long as three hours after the first titration with no change in the vitamin C content. However, extracts held for 10 to 20 hours showed a gradual reduction in the titration value indicating some loss of vitamin C.

The usual concentration of acetic acid is not recommended for the determination of vitamin C of tissue such as the banana, because the banana contains enzymes that bring about the destruction of the ascorbic acid when the tissue is macerated unless a strong acid is present to inhibit the action of these enzymes. As shown in Table I the vitamin C contents of the samples of banana macerated in 18 per cent acetic acid or in either hot or cold 8 per cent acetic acid are considerably lower than those obtained

by the use of sulphuric acid. Furthermore, the values obtained with the acetic acid are not the true values because after reduction of the extract with H_2S the values always increase indicating an oxidation of vitamin C during extraction.

Trichloracetic acid in a concentration of 8 per cent does in some cases give results comparable with those obtained with sulphuric acid. As is shown in Table I, more nearly comparable results are obtained with the ripe than with the green bananas. Only in the cases of the green bananas were there any increases in the vitamin C content after reduction with H_2S. Since the green tissue resists grinding and forms a viscous mass with the volume of trichloracetic acid used, it is possible that some oxidation takes place before the enzymes are completely inactivated.

The vitamin C values given for two lots of green bananas (see footnote of Table I) are lower than those normally found because of the exposure to the carbon dioxide which accumulated in the closed container because of the respiration of the fruit. This result has been mentioned in an earlier paper (4) and will be discussed more fully in a later paper. This reduction is not due to the effect of the dissolved carbon dioxide in the tissue extract acting on the indicator, but to an actual reduction in the total amount of ascorbic acid present in the tissue. The passage of carbon dioxide gas into banana extract will cause no change in the titration value of the dye from that obtained previous to the treatment with carbon dioxide. Also carbon dioxide dissolved in the freshly pressed juice of asparagus tissue before or after adding acid in preparation for titration with the indophenol dye, will cause no variation in the titration value from that obtained when carbon dioxide is absent. The carbon dioxide effect is not upon the extracted material but upon the living tissue previous to the extraction procedure.

The necessity of inactivating the enzymes of the banana tissue with a strong acid at the time of macerating is shown by the data in Table II. Either macerating the tissue in air or allowing the macerated tissue to stand without acid for only one minute causes a loss of as much as 50 per cent of its original vitamin C content. Likewise additional periods of time in air allow a further loss in the vitamin C content of this tissue to a point where almost complete destruction is obtained within 30 minutes. This is especially true in the case where the banana tissue is macerated, mixed with water, and oxygen passed through the mass for 30 minutes. In every case when the air-exposed macerated tissue extract was reduced with H_2S there was obtained some recovery of the oxidized vitamin C. These data indicate that the changes in the ascorbic acid from the reduced to oxidized and thence to an irreversible oxidized form are quite rapid and can be retarded or inhibited by the presence of a strong acid.

The data given in the fourth column of Table II cannot be compared with the values given in Table I since they represent the analyses of single

TABLE II

EFFECTIVENESS OF PRESENCE OF EXTRACTING ACIDS AT TIME OF MACERATING ON THE
ENZYMATIC DESTRUCTION OF VITAMIN C IN THE BANANA

Color and condition of banana (finger)	Acid medium with HPO$_3$	H$_2$S reduction of extract before titration	Vitamin C, mg. per 100 g. of fresh tissue					
			Minutes after grinding before adding acid					
			0	1	2.5	5	10	30
Yellow, green tips, firm	8% trichlor-acetic	No	12.7	5.5	4.2	4.1	—	—
		Yes	12.7	5.9	4.4	4.5	—	—
Green, hard	8% trichlor-acetic	No	16.6	—	—	—	4.8	—
		Yes	16.6	—	—	—	5.4	
Green, hard	1 N H$_2$SO$_4$	No	15.9	—	—	—	5.3	—
		Yes	15.9	—	—	—	5.3	—
Yellow, green tips, firm	1 N H$_2$SO$_4$	No	12.6	7.9	7.1	3.4	—	—
		Yes	12.6	8.5	7.6	3.6	—	—
Yellow, brown flecks, soft	2 N H$_2$SO$_4$	No	12.2	—	—	—	1.4	1.2
		Yes	12.2	—	—	—	2.4	2.2
Yellow, brown flecks, soft	2 N H$_2$SO$_4$	No	12.2	—	—	—	1.7	1.2
		Yes	12.2	—	—	—	2.5	2.2
50% brown, soft	2 N H$_2$SO$_4$	No	11.5	—	—	—	0.9*	0.9*
		Yes	11.5	—	—	—	1.4	1.3

* Macerated tissue mixed with water and oxygen bubbled through the mass.

fingers. These values in Table II do show, however, the range in the vitamin C content of the banana at different stages of ripeness.

The vitamin C content of the banana tissue was determined by both the volumetric method as already described and the extraction method of Bessey and King (1) as is usually used. In these tests both 10 and 20 gram samples of banana tissue were macerated with 30 and 50 cc. of acid respectively and made up to 100 cc. by three rinsings in the former case and to 200 cc. with three, four, and six rinsings in the latter case. In those cases when 10 grams were extracted with a total of 100 cc. of acid mixture the total value of ascorbic acid averaged 7 per cent below that obtained with the volumetric procedure. Tests with a fourth extraction of the residue in the centrifuge tube showed the presence of an appreciable amount of vitamin C still present in the mass. These operations were repeated with a 20 gram sample where the final volume was 200 cc. obtained by 50 cc. of acid for grinding and three additional extractions of the tissue. The data for this investigation are given in the first row of figures of Table III which show a loss of 10 per cent of the vitamin C content by the extraction method. Furthermore it is to be observed that there was some oxidation of the vitamin C during the extraction and none during the volumetric procedure. The next test of the extraction procedure was with

TABLE III

Comparison of Volumetric and Extraction Procedures for the Estimation of Vitamin C

Color and condition of banana (finger)	H₂S reduction of extract before titration	Vitamin C, mg. per 100 g. of fresh tissue					
		Acid medium used with metaphosphoric acid					
		Sulphuric				Trichloracetic	
		1 N		2 N		8%	
		Volumetric	Extraction	Volumetric	Extraction	Volumetric	Extraction
Green, hard*	No	12.8	10.7	—	—	—	—
	Yes	12.8	11.5	—	—	—	—
Green, hard	No	14.9	13.5	14.9	14.2	14.2	13.6
	Yes	14.9	13.6	14.9	14.2	14.2	13.7
Yellow, brown spots in peel, soft	No	11.5	11.6	11.8	11.8	11.9	11.7
	Yes	11.5	11.6	11.8	11.8	11.9	11.7
Green, hard**	No	—	—	—	—	11.2	11.0
	Yes	—	—	—	—	11.2	11.0
Green, hard*	No	12.8	12.8	—	—	—	—
	No	12.7	12.7	—	—	—	—
Yellow, firm	No	12.8	12.8	—	—	—	—
	No	12.8	12.7	—	—	—	—

* Held in air 24 hours after exposure to CO₂.
** Exposed to respired CO₂ for 48 hours.

four extractions of the tissue after macerating with 50 cc. of acid and the data are given in the second line of Table III, where is shown an appreciable loss of 4 to 9 per cent of the vitamin C content by the extraction procedure. In these tests and in others reported in this table one banana finger was sampled twice only, once for the volumetric and once for the extraction procedure for each acid medium used. Thus, it is not possible to compare the results of the volumetric procedure for the different acid media used. The remaining data in Table III show very good agreement between the volumetric and extraction method where six extractions were made of the 20 gram sample of tissue. A difference of 0.1 or 0.2 mg. of vitamin C per 100 grams of tissue is not to be considered a serious variation and is much closer than many tissues can be sampled.

Having obtained very good agreement with duplicate samples of tissue of nearly equal weight the next tests were conducted on samples of tissue varying in weight. The data in Table IV show that almost any convenient weight of banana tissue may be used, provided a sufficient quantity of a strong acid is used as a grinding medium for the extraction of vitamin C. Although no difficulty was found in grinding either a 13, 19, or 26 gram

TABLE IV

VITAMIN C CONTENT OF FINGERS AS DETERMINED BY DUPLICATE AND TRIPLICATE
SAMPLES OF LIKE AND VARYING WEIGHTS OF TISSUE

Color and condition of banana (finger)	Grams of pulp tissue from banana	Acid medium with HPO₃	Vitamin C, mg. per 100 g. of fresh tissue	
			H₂S reduction before titration	
			No	Yes
Yellow	20.799 20.971	1 N H₂SO₄	12.3 12.3	12.3 12.3
Yellow	20.267 20.715	1 N H₂SO₄	12.4 12.4	12.4 12.4
Yellow, brown flecks	18.753 18.837	1 N H₂SO₄	12.8 12.8	12.8 12.9
Green	20.635 20.712 19.178	2 N H₂SO₄	15.2 15.1 15.2	15.2 15.1 15.2
Green	11.640 16.459 19.677	2 N H₂SO₄	15.6 15.5 15.7	15.6 15.5 15.7
Green	19.575 26.223 13.668	2 N H₂SO₄	15.8 15.6 15.7	— — —

sample of tissue from the same banana finger with 50 cc. of 2 N sulphuric acid mixture and obtaining close agreement in the vitamin C content, it is recommended that if larger samples are used more of the acid mixture must be present at the time the tissue is macerated.

A comparison of the vitamin C data in Table IV before and after reduction with H₂S shows that reduction of the strong acid extracts before titration with the dye is unnecessary. When the correct acid or strength of acid is determined with the volumetric procedure for any particular tissue the reduction of the extract with hydrogen sulphide becomes an unnecessary time-consuming part of the procedure in the determination of vitamin C.

Duplicate samples of banana tissue were macerated, one with 5 and the other with 10 grams of quartz sand in 1 N sulphuric acid and the vitamin C content of the two samples was found to be identical. This result shows that no appreciable error is introduced by the addition of this quantity of sand to the tissue and washing it into the volumetric flask. As a result of various tests where the correction was made for the sand added for grinding there was an average difference of only 1.4 per cent in the vitamin C content of the tissue. If, for example, a tissue containing 16 per cent insoluble matter was washed into the volumetric flask

with the sand the total error introduced by both materials would be only 2.4 per cent in the final value for the vitamin C content of the tissue. This error is, in many cases, much smaller than that introduced into the extraction method; furthermore, there is the assurance of a representative sample of all of the vitamin C in the tissue in an unoxidized form if sufficiently strong acid is used.

DISCUSSION

The volumetric procedure here proposed has the advantage over the extraction procedure now in use in that a representative sample of the total vitamin C content of the tissue is obtained with one maceration and centrifuging of the suspension of this macerated tissue. Furthermore, if sufficiently strong acids are used during the grinding process no additional treatment of the extract with H_2S is necessary. With the extraction procedure approximately twice as much time is involved in obtaining the separation of the vitamin C from the tissue and considerable care must be taken to insure that a sufficient number of extractions of the tissue have been made to separate completely the vitamin C from the tissue. The number of extractions of the plant tissue usually recommended after macerating with acid are not sufficient to obtain all of the vitamin C of the banana tissue. However, the values for the vitamin C content obtained with six extractions of the macerated tissue are comparable with the values obtained by the volumetric procedure that gives the vitamin C content with only one process of separating the liquid from the suspended matter.

Extreme care must be exercised in the choice of the acid used in the extracting medium for the determination of vitamin C. Where there are no ascorbic acid oxidizing enzymes present in the plant tissue to interfere with the determination, the concentration of acetic acid usually recommended may be used. However, in the case of the banana 8 or 18 per cent acetic acid gives low values and in some cases the use of 8 per cent trichloracetic acid is to be questioned. The safest procedure is to use 1 N, 2 N, or 3 N sulphuric acid which have no detrimental effect upon the vitamin C content of the tissue and not only protect the vitamin C from enzyme action but also make unnecessary a H_2S treatment for recovery of oxidized ascorbic acid. The acid mixture must contain the required amount of metaphosphoric acid to minimize the action of copper in the solution.

SUMMARY

1. The successive extraction procedure usually recommended for removing vitamin C (ascorbic acid) from macerated plant tissue is not satisfactory because several (at least more than three) extractions are necessary for its complete removal.

2. This paper describes a modification of this method which requires only one maceration and extraction of the tissue. The modified procedure is accurate and requires considerably less time and manipulation to prepare the tissue extract for titration with the solution of 2.6-dichlorophenolindophenol.

3. In the banana there are enzymes present which bring about a rapid destruction of the vitamin C content when the tissue is macerated in air, reducing the ascorbic acid values by 50 per cent within one minute if sufficient concentration of the proper acids is not present at the start of the maceration.

4. Only strong acids such as 1 N, 2 N, or 3 N sulphuric acid in combination with $N/4$ metaphosphoric acid which quickly inactivate the enzymes of the tissue were found satisfactory in the separation of vitamin C from tissues containing the ascorbic acid oxidizing enzymes.

5. Neither 8 nor 18 per cent acetic acid, nor, in some cases, 8 per cent trichloracetic acid, is of sufficient strength to inactivate the enzyme, and on this account when such acids are used the values obtained were too low.

6. The use of suitable concentrations of sulphuric acid eliminated the necessity of treating the tissue extract with hydrogen sulphide to recover oxidized ascorbic acid.

Note Added in Galley Proof

Since the preparation of this paper tests have shown that complete reduction of oxidized ascorbic acid with hydrogen sulphide is inhibited by strong acid solutions such as recommended for the extraction. This result does not require any modification of the recommended procedure. The strong acid mixture employed inhibits oxidation and no reduction of oxidized ascorbic acid is needed. Furthermore tests show that ascorbic acid added to banana tissue at the time of grinding may be completely recovered without the necessity of the reduction with hydrogen sulphide.

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SUGGESTIONS FOR STANDARDIZING THE ETHYLENE CHLORHYDRIN TREATMENT FOR INDUCING SPROUTING OF RECENTLY-HARVESTED INTACT POTATO TUBERS[1]

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The problem is to find the approximate conditions for treating the tubers so that sprouting will be initiated even in the uncut tubers before planting, and to have a method of assuring oneself within a day or two, or at most within five days after the start of the treatment, that the treated tubers have been brought into this condition.

Previous experiments (2) showed that the quantity of ethylene chlorhydrin found in an unaltered condition in potato tubers at the end of a five-day period of treatment was correlated with the subsequent growth of the tubers after they had been cut and planted; and that the range for inducing a satisfactory gain in germination was quite wide, i.e., from a lower limit of about 1.0 cc. to an upper limit of 15 or 20 cc. of 0.1 M ethylene chlorhydrin per 100 g. of tissue, or even more, depending upon the temperature during the treatment.

In the experiments here reported, samples were taken each day during the five-day treatment, these providing information as to the progress of the absorption of the chemical, and as to the duration of the treatment necessary to hasten germination satisfactorily. Two varieties, Bliss Triumph and Irish Cobbler, were used, and both of these at three intervals of one week each after harvest.

The results indicate a rather wide range of conditions under which breaking of dormancy may be expected to occur, and show that a chemical test, not too complicated to be feasible, at least when large quantities of tubers are to undergo treatment, may be used to determine the time at which the treatment may be discontinued, i.e., when the tubers have been brought into condition for subsequent sprouting.

METHODS

The potato (*Solanum tuberosum* L.) tubers used in the main tests were harvested in the interval from August 2 to 5, 1937, and were spread on the floor indoors for one week before the treatments were started. In later tests a crop from late-planted tubers was harvested October 22, 1937, and tests with these were started in the interval from the first to the fourth day after harvest.

The treatments were made in 2-gallon glazed earthenware jars, using 28 to 40 tubers (weighing 2500 to 3500 g.) in each jar. The ethylene chlorhydrin (CH_2ClCH_2OH) was taken up in a piece of cheesecloth which was spread loosely in a large watch-glass placed over the tubers in the jar. Modeling clay was used for sealing. The ethylene chlorhydrin solution used was the commercial 40 per cent solution, 1 cc. of which is equivalent to approximately 50 cc. of 0.1 M ethylene chlorhydrin.

After the treatment the tubers were not planted at once, but were divided into two lots and stored at room temperature in paper bags, one lot being planted 4 days, the other lot 14 days, after the end of the treatment. Before planting the tubers were cut into one-eye pieces weighing approximately 25 grams each, 50 pieces constituting a sample. They were planted in soil in flats stored in tiers indoors at room temperature (approximately 22° C.). Each 4 to 7 days a count was made of the emerged sprouts. The criterion used for representing the data was the time after planting for 90 per cent emergence, this end-point representing not only rate but also completeness of germination. Procedures that did not produce 90 per cent germination were considered undesirable whatever might be the rate for, let us say, 50 per cent. Procedures that induced 90 per cent emergence in about 30 days from planting were regarded as successful.

The method used for the determination of the ethylene chlorhydrin absorbed during the treatments is essentially the same as was previously used (2) except that the determinations were made on the expressed juice rather than on the tissue. The method is based on the fact that ethylene chlorhydrin forms a constant boiling mixture with water (of minimum boiling point) and can therefore be recovered by distillation. The ethylene chlorhydrin distilled over is allowed to react with $Ba(OH)_2$ to form $BaCl_2$. The chloride formed can then be titrated by the Volhard method which involves the addition of an excess of $AgNO_3$ solution after acidification with HNO_3 and titration of the excess $AgNO_3$ with potassium thiocyanate, using ferric ammonium sulphate as an indicator. Previous tests with this method for chlorhydrin have shown that from 85 to 90 per cent of added chlorhydrin is recovered.

For the determination of the chlorhydrin absorbed in a given treatment it is important to take special care to get a representative sample since there is considerable variation in the amounts absorbed by individual tubers. In the experiments reported in this paper each determination was based on a sample obtained from one-half of the tubers used in the treatment. One-quarter was removed from each tuber sampled and the juice obtained by grinding through a food chopper with a fine cutter and subsequently squeezing by hand through cheesecloth. The juice was allowed to stand for a short time to permit the starch to settle out and then aliquots were taken for distillation. It is somewhat more convenient to use

juice than tissue since it is easier to get a representative sample and the distillation proceeds more smoothly. Comparisons made on the quantities of chlorhydrin obtained when determined in terms of 100 cc. juice and 100 g. tissue have shown that when expressed in terms of juice the results are somewhat higher. An average of 15 such comparisons has shown that in order to convert results expressed in terms of the juice to tissue one should multiply by the factor 0.81.

Quantities of juice varying from 100 to 200 cc. were used for the distillation. Water was added to make a total volume of 250 cc. In addition 3.3 cc. of N H_2SO_4 were added for each 100 cc. of juice. The distillate was collected in a 200 cc. volumetric flask containing 9 grams of $Ba(OH)_2$. An aliquot of the solution in $Ba(OH)_2$ was titrated by the Volhard method after standing overnight. If the $Ba(OH)_2$ solution is heated to boiling the titration can be made at once after cooling and thus the whole determination may be completed in a few hours if it is desired to have a prompt answer as to the amount of chlorhydrin absorbed in a given treatment.

RESULTS

ABSORBED ETHYLENE CHLORHYDRIN AND SPROUTING RESPONSE

The results are shown in Table I. In column 2 are listed the amounts of ethylene chlorhydrin applied per 100 g. of tubers at the start of the treatment, 20 cc. of 0.1 M representing the upper limit, and 5 cc. the lower limit for treatment, as shown by previous tests.

If we consider that a successful treatment is one in which not more than about 30 days are required for 90 per cent sprout emergence, and if we compare the successes and failures with the amounts of ethylene chlorhydrin found in the corresponding juice samples, as shown in columns 4, 7, and 10, we find that a safe range for success under all of the conditions of the test is from 8 to 12 cc. of 0.1 M ethylene chlorhydrin per 100 cc. of juice. When such amounts of ethylene chlorhydrin were found in the press-juice, good sprouting invariably resulted with both varieties whatever the temperature of the treatment (within the limits that occurred in the tests) whether the treatment lasted one day or five, and whether the treatment was applied at the first, second, or third week after harvest. Favorable results were obtained at amounts much lower than 8 cc.; indeed, a 5 cc. minimum gave 17 successes out of 18, and the range 3.5 cc. to 5.0 cc. showed 9 successes out of 12. But when the amounts absorbed were below 3.5 cc. of 0.1 M ethylene chlorhydrin per 100 cc. of juice 21 failures out of 31 resulted. In the higher range, 12 to 15 cc. gave 7 successes and no failures, and while 15.7 cc. gave injury in one test, other lots showed sprouting and no rotting of seed-pieces at 15.2, 17.3, 17.5 and 18.2 cc.

Although 30 days for 90 per cent germination was taken as a criterion

TABLE I

RELATIONSHIPS BETWEEN AMOUNTS OF ETHYLENE CHLORHYDRIN USED, ABSORBED BY TISSUE, TEMPERATURE, DURATION OF TREATMENTS, AND TIME FOR EMERGENCE OF SPROUTS OF DORMANT POTATO TUBERS

Variety	Cc. 0.1 M chlorhydrin per 100 g. tubers	Duration of treatment, days	Treated 1 week after harvest. Temp. 27° C.			Treated 2 weeks after harvest. Temp. 26° C.			Treated 3 weeks after harvest. Temp. 22° C.		
			Cc. 0.1 M chlorhydrin per 100 cc. juice	Days for 90% emergence. Planted days after treatment		Cc. 0.1 M chlorhydrin per 100 cc. juice	Days for 90% emergence. Planted days after treatment		Cc. 0.1 M chlorhydrin per 100 cc. juice	Days for 90% emergence. Planted days after treatment	
				4	14		4	14		4	14
Bliss Triumph	20	1	11.9	17	9	9.5	13	14	2.8	48	28
		2	13.1	15	13	14.3	13	12	4.5	15	24
		3	12.6	15	—	18.2	15	8	5.8	25	11
		4	13.3	19	11	13.3	16	11	6.5	13	10
		5	15.7	*	*	17.5	13	6	8.4	12	9
	10	1	7.3	11	9	6.7	13	14	2.1	40	46
		2	6.2	12	13	9.3	11	12	2.6	38	39
		3	8.1	9	12	6.3	13	8	4.7	14	12
		4	7.3	11	11	6.1	12	11	4.3	22	12
		5	6.4	21	16	5.4	13	6	2.5	27	14
	5	1	3.4	29	14	3.5	15	23	1.0	40	40
		2	3.8	15	13	3.4	13	9	1.6	24	38
		3	3.6	9	15	3.0	14	11	1.6	39	43
		4	2.8	17	11	3.3	14	9	1.4	25	31
		5	1.2	21	40	1.9	16	9	1.1	34	26
	Control		0	75	63	0	67	54	0	58	44
			Temp. = 26° C.			Temp. = 22° C.			Temp. = 24° C.		
Irish Cobbler	20	1	8.8	21	12	2.8	54	57	3.5	18	33
		2	13.2	18	7	5.5	26	65	5.7	16	21
		3	17.3	19	9	2.5	45	29	9.7	13	13
		4	15.2	12	8	4.4	15	16	9.9	19	14
		5	14.7	17	18	6.6	13	18	4.3	21	24
	10	1	6.4	32	16	1.7	69	60	2.3	18	16
		2	7.3	19	12	2.2	64	66	3.7	12	21
		3	6.6	24	20	3.1	38	43	4.3	13	37
		4	5.1	10	8	2.4	43	53	2.2	34	25
		5	7.0	13	11	2.1	50	42	3.9	55	24
	5	1	2.1	38	47	1.5	67	68	1.7	37	32
		2	2.9	34	23	1.8	60	66	1.4	23	32
		3	4.2	23	17	1.5	64	36	1.9	56	37
		4	2.7	22	27	1.1	63	63	0.7	77	48
		5	1.5	31	32	0.3	63	41	1.5	57	54
	Control		0	86	66	0	72	63	0	59	62

* 90% emergence not obtained because of rotting of seed-pieces.

for success, and although it is stated in the preceding paragraph that values below 3.5 cc. showed a high percentage of failure, even in this range shortening of the time for germination was obtained; thus, when the temperature was higher than 22° C. the range from 2.0 to 3.5 cc. of absorbed chlorhydrin showed average gains of 40 to 50 days over the time required for 90 per cent germination of control tubers; but at 22° amounts below 3.0 cc. showed very little gain over the controls, either because of the natural shortening of the dormant period, as with Bliss Triumph (3rd week), or because of the ineffectiveness of the treatment with these small amounts of absorbed chemical, as with Irish Cobbler (2nd week).

<h3 style="text-align:center">DURATION OF TREATMENT AND QUANTITY OF UNALTERED CHEMICAL IN TISSUE</h3>

The quantity of chemical found unaltered in the tissue did not increase gradually from day to day as one might expect. The values in columns 4, 7, and 10 show surprisingly uniform amounts in the tissue for any given treatment whether the duration was one day or five. With the treatments at 26° and 27° C. approximately two-thirds of the available chemical had been taken up by the end of the second day, and consequently further large increases could not be expected. Another reason for not finding larger quantities of chemical in the tissue toward the end of the tests is that a continuous decomposition of the chemical was taking place, as shown in the reports of previous experiments (1, p. 177). This may explain why in some of the tests there was a falling off in the quantity of chemical found in the tissue on the fifth day of treatment, since probably nearly all of the available chemical had been absorbed by the tissue during the previous days, and the loss by decomposition of the chemical within the tissues could not be replaced by further accretions from the surrounding air.

With treatments at 22° and 24° there was some evidence of increasing quantities of chemical in the tissues with increasing duration of treatment, particularly for treatments with the highest amount of chemical. But there was no evidence in these cases that extending the period of treatment would have been of importance for successful treatment. If about 5 cc. of 0.1 M per 100 cc. of juice were not present in the tissue by about the third day, longer treatments were not effective.

<h3 style="text-align:center">QUANTITY OF ETHYLENE CHLORHYDRIN ABSORBED BY CUT POTATO TISSUE IN DIP TREATMENTS</h3>

These results, showing that whole tubers should take up about 8 to 12 cc. of 0.1 M ethylene chlorhydrin in order to be in condition for subsequent germination, raised the question as to the amounts of chemical absorbed by the tissue in the "dip" treatments previously recommended (1, p. 161) for use with tubers that had been cut into pieces and which

were treated and planted at once after treatment. By this method the cut pieces (approximately 25 grams each) are dipped momentarily into a dilute solution of ethylene chlorhydrin and are then stored in a closed container for 24 hours before being planted. Previous experiments had shown that for the dipping solution in such treatments, 50 cc. of 40 per cent ethylene chlorhydrin made up to 1 liter with water represented approximately the optimum concentration, that 100 cc. were the upper limit, that 25 cc. were the lower limit, and that 12.5 cc. produced a dipping solution that was too dilute. A test was made to find the quantity of ethylene chlorhydrin present in the press-juice from tissue treated in this way in order to compare with the results obtained from the whole-tuber tests. The results are shown in Table II. The tissue treated with the dip-

TABLE II

QUANTITY OF ETHYLENE CHLORHYDRIN IN POTATO TISSUE AFTER TREATMENT
BY THE "DIP" METHOD FOR CUT TUBERS

Cc. of 40% ethylene chlor-hydrin used in the preparation of the dipping solution	Cc. of 0.1 M ethylene chlor-hydrin per 100 cc. of juice from the tissue after treatment
100	16.2
50	8.3
25	4.3
12.5	2.1

ping solution previously found to be optimum for sprouting (50 cc.) gave a press-juice with a titration value of 8.3 cc., and the upper and lower limits in this test were 16.2 cc. and 2.1 cc., respectively, values nearly identical with the values shown in Table I for the press-juices from the whole-tuber treatment. The whole-tuber and "dip" methods agree in indicating that potato tissue that has taken up sufficient ethylene chlorhydrin to give a titration value of about 10 cc. of 0.1 M ethylene chlorhydrin per 100 cc. of press-juice is in condition for subsequent germination.

THE EFFECT OF TEMPERATURE

The values in Table I show a considerable effect of the temperature prevailing during the treatment upon the absorption of the chemical and upon the subsequent growth. At 22° the amount and rate of absorption were both much reduced, and in many of the tests with the smaller amounts of chemical not enough chemical was taken up to get within the optimum range. Further experiments are needed on this phase of the problem.

TREATMENTS DURING THE FIRST FEW DAYS AFTER HARVEST

Although most of the tests were carried out upon tubers that had been harvested at least one week previous to treatment, a few lots were treated

within the first week interval after harvest. Tubers for these tests were from a late planted crop, harvested October 22 in an immature condition, the tubers being small and weighing 36 grams each. The treatments of Bliss Triumph tubers were started the day following harvest, and those for the Irish Cobblers on the fourth day after harvest. In both cases the

TABLE III

TREATMENTS OF POTATO TUBERS DURING THE FIRST FEW DAYS AFTER HARVEST

Variety	No. days after harvest until treatment started	Duration of treatment, days	Temperature		Days for 90% emergence
			During treatment	During 10 days' storage after treatment	
Bliss Triumph	1	1	Room	Room 27° C.	47 35
			27° C.	Room 27° C.	40 30
		2	Room	Room 27° C.	43 38
			27° C.	Room 27° C.	* *
		Controls	Room	Room 27° C.	73 66
			27° C.	Room 27° C.	71 66
Irish Cobbler	4	1	Room 27° C.	27° C. "	51 47
		2	Room 27° C.	" "	17 21
		4	Room 27° C.	" "	24 *
		Controls	Room 27° C.	" "	96 94

* 90% emergence not obtained because of rotting of seed-pieces.

amount applied at the start of the treatment was 20 cc. of 0.1 M ethylene chlorhydrin per 100 g. of tubers. Since previous tests (2, p. 129) had shown that freshly-harvested tubers were quite permeable to the chemical vapors the duration of the tests was reduced to not more than four days and to intervals as short as one day only. Treatments were made both at room temperature and at 27° C. After treatment the tubers were stored in paper bags at two different temperatures, room temperature and 27° C. The results are shown in Table III.

Treatments for periods as short as one and two days when applied even in this early period after harvest shortened the rest period by 26 to 79 days, as shown by comparing the treatment values in column 6 with those for the corresponding controls. Storage at 27° C. for a period of 10 days after treatment gave gains over room temperature storage for the same period, but a temperature of 27° C. during the period of treatment caused rotting of seed pieces if the duration was as long as two days for the Bliss Triumph or four days for the Irish Cobbler tubers.

The cause of the injurious effect of the high temperature during the treatment period probably is to be found in the lack of oxygen resulting from the high respiration induced both by the chemical treatment (3) and by the high temperature itself. Lack of injurious effect by the high temperature during storage after treatment is probably related to the good aeration furnished to the treated tubers after their removal from the containers.

Further experiments on tubers during the first few days after harvest with special attention to the amounts of ethylene chlorhydrin found in the press-juice from the treated tubers are needed in order to determine whether treatments can be made safely at this early period.

SUGGESTIONS FOR LARGE SCALE TREATMENTS

If the treatment is upon a scale sufficiently large to justify the employment of a person with sufficient chemical training to carry out the titrations indicated in the section under "Methods," the end-point of the treatment can be determined accurately, and considerable confidence may be had in the capacity of the treated tubers to germinate when planted later. In this case the room in which the treatment is applied need not be absolutely air-tight but should be sufficiently tight to permit the tubers to have a chance to absorb the chemical vapors. The chemical can be evaporated from burlap bags suspended in the room. For the start of the treatment the amount of chemical should be one quart of 40 per cent ethylene chlorhydrin (the usual commercial material) for each 12.5 bushels of tubers. The room should be closed for two days if the average temperature for the period is 75° F. or more, or for three days if it is less than 75° F. Then the room should be opened, and thoroughly aired, after which a sample of at least 200 tubers should be taken for analysis, care being taken that this sample consists of tubers from various localities in the room. One lengthwise quarter of each of these tubers is taken for a combined sample, the tissue passed through a food-grinder, and juice squeezed out by putting the ground tissue in a cheesecloth bag, and twisting. A sample of 100 to 200 cc. of juice is taken for the distillation as shown in the paragraph under "Methods." If the titration value is 8

cc. of 0.1 M per 100 cc. juice or more, the treatment is completed. If the value is less than this another treatment for one or two days should be applied in the same way, using one quart to each 12.5 bushels of tubers if the value was less than 5 cc., and one quart to each 25 bushels if the value was between 5 and 8 cc. Another sample and another distillation will show whether the proper amount of chemical has been absorbed. If the final value is more than 15 cc. the tubers should be distributed in such a manner as to give good aeration, and should be held for observation for about 10 days in order to see whether injury to the tubers has occurred.

Previous tests (1, p. 167) have shown that it is undesirable that tubers treated in this way be planted at once after the treatment. More favorable results are obtained if the planting is delayed about four days. The intact tubers stored in air show initiation of sprouts in about one week. The present tests show that not much time was lost if the tubers were not cut and planted until two weeks after treatment.

The vapors of ethylene chlorhydrin are poisonous if breathed in high concentration for extended periods, but under the conditions described in this article the amount of vapor in the room at the end of the treatment after aeration will be small, and the removal of tubers from the room can be arranged so that prolonged breathing of vapors can be avoided.

If the treatment is not upon a scale justifying the employment of a chemist for the titration referred to under "Methods," the end-point of the treatment cannot be determined with such dependability. Nevertheless, the tests indicate the approximate conditions for the treatments. At average temperatures of 75° F. to 80° F. use one quart of 40 per cent ethylene chlorhydrin for each 12.5 bushels of tubers and maintain the contact for three days; at temperatures of 70° F. to 75° F. use one quart for each 9 bushels of tubers and maintain the contact for five days.

These suggestions apply to tubers in the period one to three weeks after harvest from vines just dead at the time of harvest. As for the treatment of tubers within the first week after harvest, or for treatments at temperatures above 80° F. or below 70° F., not enough information has been obtained to justify a recommendation.

SUMMARY

Tubers of two varieties of potatoes (*Solanum tuberosum* L.), at intervals of one, two, and three weeks after harvest, at temperatures of 22° to 27° C. were treated with varying amounts of ethylene chlorhydrin (CH_2ClCH_2OH) for periods of one to five days. Samples of tubers at the end of each treatment were taken for obtaining press-juice to show the amount of ethylene chlorhydrin present in the tissue. The sprouting re-

sponse of samples from each lot was compared with the corresponding amount of absorbed ethylene chlorhydrin and with the experimental conditions leading to the absorption of that amount of chemical.

The results show that the quantity of ethylene chlorhydrin that had been absorbed by the tissue was a dependable indicator of subsequent sprouting; that when about 8 to 12 cc. of 0.1 M ethylene chlorhydrin per 100 cc. of press-juice were found in the tubers at the end of a treatment such tubers gave a successful growth response (90 per cent emergence within about 30 days from planting) whatever the temperature during the treatment, whether the treatment lasted one day or five, and whether the treatment was applied at the first, second, or third week after harvest; and further, that favorable responses were usually obtained when the amounts of absorbed ethylene chlorhydrin were as low as 5 cc. and as high as 15 cc. of 0.1 M.

A procedure is suggested for large scale treatments of dormant tubers. It is based on the removal of samples of tubers at intervals after the start of the treatment, obtaining press-juice, distilling the juice, and determining the absorbed ethylene chlorhydrin by titration. In this way the time at which the tubers have been brought into condition for subsequent sprouting may be determined.

For the treatment of tuber quantities too small to justify the use of the distillation procedure, suggestions are made as to the amount of chemical to be applied and the duration of the treatment at different temperatures.

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PHOSPHATASE ACTIVITY OF THE JUICE OF PLANT TISSUE FOLLOWING TREATMENT WITH ETHYLENE CHLOROHYDRIN[1]

John D. Guthrie

Since ethylene chlorohydrin has been shown to have such a marked effect on the catalase, peroxidase, and dehydrogenase content of recently-harvested potato tubers (2) it is planned to extend the investigation to other enzymes. Attention was directed to phosphatase by two conflicting papers that appeared about the same time. One by Ignatieff and Wasteneys (3) reported very low values for phosphatase in potato tubers, while the other by Pfankuch (4) reported high values. The first step in the present investigation was to compare the procedures used by these workers. It was found that this did not account for the widely different results. For example: Ignatieff and Wasteneys peeled the tubers and worked on dried tissue, but it was found that drying did not destroy the phosphatase nor did peeling have much effect. They also used mother-tubers, but experiments showed that these contained about as much phosphatase as other tubers from the same lot. The results obtained in the present work show that potato tubers contain phosphatase, but the values obtained range from 25 to 65 PE per cc., which is lower than the range of 100 to 200 PE per cc. given by Pfankuch. It appears that potato tubers vary widely in phosphatase content and that this explains the conflicting results cited above.

Ethylene chlorohydrin treatments were found to decrease the phosphatase activity of the juice of whole, freshly-harvested potato tubers, and this was found to be due to the formation of an inhibiting substance which was removable by dialysis. This inhibitor may be of some interest because of the work of Thannhauser, Reichel, Grattan, and Maddock (5) who have called attention to the importance of inhibitors of phosphatase in the treatment of Paget's disease. The effect of ethylene chlorohydrin on the phosphatase activity of the juice of gladiolus corms and of Jerusalem artichokes was also investigated, but no inhibition was found.

METHODS AND RESULTS

In general, phosphatase was determined by the procedure used by Pfankuch (4), except that Briggs' reagents (1) were used in the determination of the phosphate liberated by the enzyme, rather than the sodium sulphite, aminonaphtholsulphonic acid, potassium metabisulphite

mixture. The Briggs reagents were more stable and gave a more stable color. In several experiments the reagents were compared and essentially the same phosphatase values obtained. The juice was from unpeeled tissue, but where cut pieces were used, the callus was removed in a thin layer. Potato juice was usually diluted one-fifth and 2 cc. of the diluted juice added to 10 cc. of the substrate in a bath at 30° C. After one hour 10 cc. of 6 per cent trichloroacetic acid was added to stop the reaction and the solution filtered. Two cc. aliquots were placed in 25 cc. volumetric flasks, 10 cc. water and 2 cc. of the three Briggs reagents added in proper order. These reagents are: (1) 5 per cent ammonium molybdate in 5 N H_2SO_4; (2) 1 per cent hydroquinone plus one drop H_2SO_4; (3) 20 per cent sodium sulphite. At the same time standards were prepared containing from 0.05 to 0.2 mg. phosphate phosphorus and the determinations compared colorimetrically with these after standing at least one-half hour. The substrate was prepared by mixing 11 g. sodium-β-glycerophosphate, 189 cc. H_2O and 24 cc. N/1 HCl. The pH was 5.75. The results were calculated to PE (Phosphataseeinheit), which is the amount of phosphatase yielding 0.1 mg. of phosphate phosphorus from sodium-β-glycerophosphate in one hour.

The effect of ethylene chlorohydrin on the phosphatase content of potato tubers (*Solanum tuberosum* L.), gladiolus corms (*Gladiolus* sp. var. Alice Tiplady) and Jerusalem artichoke tubers (*Helianthus tuberosus* L.) is shown in Table I. In the case of the vapor method, whole tubers were treated with 3 cc. of 40 per cent ethylene chlorohydrin per kg. for 24 hours in containers half-filled with tubers. In the dip method, cut pieces were dipped in ethylene chlorohydrin, 40 cc. of 40 per cent per liter and stored in a closed container for 24 hours. The Jerusalem artichokes were also treated by the dip method. The gladiolus corms were treated with 1 cc. of 40 per cent ethylene chlorohydrin per 100 g. of corms for 4 days. The check sample was kept in a closed container for 4 days. The potatoes used were second crop Irish Cobbler tubers harvested in New Jersey a few weeks before treatment and new Bliss Triumph tubers from Florida. The gladiolus corms and Jerusalem artichoke tubers were from the Institute garden. It will be seen that whole tubers showed a larger and quicker decrease in phosphatase than cut tubers. In the case of the experiment with Bliss Triumph tubers the decrease in phosphatase was small. Table I also shows that a small increase in phosphatase resulted from ethylene chlorohydrin treatments of gladiolus corms and Jerusalem artichokes.

In order to check further the inhibition of phosphatase by ethylene chlorohydrin treatments and to see if this was due to an actual decrease in the enzyme or to the formation of an inhibiting substance, dialysis of the juice was tried. The juice, 20 cc., was dialyzed for three hours against

TABLE I

EFFECT OF ETHYLENE CHLOROHYDRIN ON THE PHOSPHATASE CONTENT OF POTATO TUBERS, GLADIOLUS CORMS, AND JERUSALEM ARTICHOKE TUBERS

Material used	Method of treatment	Condition of tubers or corms after treatment	Days after treatment	Phosphatase in PE per cc. juice	
				Treated	Check
Potato tubers, Irish Cobbler	Vapor	Whole	3	16	50
			6	36	51
			10	30	52
			13	13	44
		Cut	3	52	57
			6	52	52
			10	48	56
			13	36	52
	Dip	Cut	3	47	45
			6	46	45
			10	44	48
			13	36	43
Potato tubers, Irish Cobbler	Vapor	Whole	5	37	52
Potato tubers, Irish Cobbler	Vapor	Whole	1	52	58
			7	14	47
Potato tubers, Bliss Triumph	Vapor	Whole	5	27	34
Gladiolus corms, var. Alice Tiplady	Vapor	Whole	6	55	47
			12	76	50
Jerusalem artichoke tubers, red variety	Dip	Cut	3	55	54
			6	62	53
			13	64	51
Jerusalem artichoke tubers, white variety	Dip	Cut	3	58	58
			6	69	57
			13	67	52

running tap water and then diluted to 100 cc. The juice that had not been dialyzed was also diluted 20 cc. to 100 cc. Phosphatase was determined on 2 cc. of the diluted juice. The potatoes used were new, second crop, Irish Cobbler tubers from New Jersey, old Irish Cobbler tubers from the local market, and new Bliss Triumph tubers from Florida. They were treated by the vapor method, 3 cc. of 40 per cent ethylene chlorohydrin per kg. for 24 hours. The results given in Table II show that with the exception of the old tubers, the treatments brought about a decrease in the phosphatase activity of the juice. Dialysis of juices showing this decrease always increased the phosphatase activity, and frequently raised to it the level of the juice of untreated tubers. The results show that the decrease in phosphatase activity following treatment with ethylene chlorohydrin is due to the formation of an inhibiting substance which can be removed by dialysis. The boiled, filtered juice of treated tubers had no inhibiting

TABLE II

EFFECT OF DIALYSIS ON THE PHOSPHATASE OF THE JUICE OF ETHYLENE
CHLOROHYDRIN TREATED POTATO TUBERS

Tubers used	Days after treatment	Phosphatase in PE per cc. juice			
		Not dialyzed		Dialyzed	
		Treated	Check	Treated	Check
Irish Cobbler, new	5	31	50	48	49
	11	34	50	44	47
	19	24	48	29	48
	1	59	66	56	64
	2	61	65	60	66
	7	34	52	47	50
Irish Cobbler, old	8	24	27	23	25
Bliss Triumph	5	21	40	32	38

action on the phosphatase activity of the juice of untreated tubers nor on dialyzed juice of untreated tubers. This indicates that the inhibiting substance is destroyed or removed by boiling the juice and filtering out the coagulated proteins. In some experiments in which the tubers were given anaerobic conditions by covering with paraffin or placing in nitrogen, a large decrease in phosphatase was observed. In this case, however, dialysis did not restore the phosphatase activity. The boiled, filtered juice had no inactivating effect.

Ethylene chlorohydrin, ethylene glycol, glycerol, ethyl alcohol, acetaldehyde and glutathione had no effect on the phosphatase activity of the juice of untreated tubers. Glutathione was tried under a variety of conditions since Thannhauser, Reichel, Grattan, and Maddock (5) have shown that it inactivates serum phosphatase. Pfankuch (4) has pointed out that glutathione does not inactivate potato phosphatase which has a pH optimum of 5.85, while it does inactivate serum phosphatase the optimum of which is about pH 8.9.

During the course of the investigation the phosphatase content of the juice of some other plants has been determined. The results, expressed as PE per cc., are as follows: tomato (*Lycopersicon esculentum* Mill.) leaves 20, stems 17; bean (*Phaseolus vulgaris* L.) leaves 194, stems 79; tobacco (*Nicotiana tabacum* L.) leaves 34, stems 17; potato (*Solanum tuberosum* L.) leaves 37, stems 31. The high value for bean is in agreement with the results of Ignatieff and Wasteneys (3).

SUMMARY

The phosphatase activity of the juice of whole, recently-harvested potato tubers treated with ethylene chlorohydrin was lower than that of

control tubers. This was due to the presence of an inhibiting substance in the treated juice which could be removed by dialysis. Small increases in phosphatase activity of the juice of gladiolus corms and Jerusalem artichoke tubers were observed following treatment with ethylene chlorohydrin.

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TROPIC RESPONSES OF LEAFY PLANTS IN-
DUCED BY APPLICATION OF
GROWTH SUBSTANCES

P. W. ZIMMERMAN AND A. E. HITCHCOCK

The results of recent experiments indicate that synthetic growth sub-
stances applied to tips or bases of leafy shoots were unequally distributed
through the stems and leaves according to the relative position of these
organs to gravity. The direction and degree of induced bending (negative
or positive geotropism) of stems placed in a horizontal position were de-
pendent upon the concentration of the applied growth substance. Meas-
urements by means of surface markings indicated that growth was ac-
celerated regardless of whether the stem showed a negative or positive
geotropic response. The present concepts of tropisms do not afford a satis-
factory explanation for all of these induced geotropic responses.

Under normal conditions the organs of a plant maintain a certain
position of equilibrium with respect to the earth. When the organs are
shifted away from the normal relative position or exposed to one-sided
illumination, curvatures appear. Such responses are known as geotropic
when caused by gravity and phototropic when caused by light. Bending is
probably due to unequal rates of growth on the two sides of the organ,
the slow-growing side becoming the concave and the more rapidly grow-
ing the convex side. The cause of the different rates of growth on the two
sides of a stem has been the subject of many scientific investigations ex-
tending back to the 17th century. Many controversial views were held
before the modern growth substance concepts were established. In order
to present a brief historical picture, a few of the outstanding contributions
from the time of Darwin are cited.

Darwin (15) in 1881 showed that the coleoptiles of *Phalaris* and *Avena*
curved toward the light and that when only the tip was unilaterally il-
luminated the influence traveled downward. When the tip was shaded
the remaining stump was unable to make a phototropic response. This
definitely showed that the tip of the coleoptile was a place of great im-
portance in connection with phototropic curvatures in plants, though
Darwin did not recognize the influence as of a chemical nature.

The beginning of the chemical substance idea with proof to support
it goes back to 1907 when Boysen-Jensen (2, 3, 4) started his classic ex-
periments to show that the stimulus (chemical substance) could cross a
discontinuity in the coleoptile of *Avena*. He found that when excised
coleoptile tips were replaced on the stump with a layer of gelatin, photo-
tropic curvatures resulted after unilateral illumination of the tip as with

normal coleoptiles. That is, a substance which was formed in the tip drained into the gelatin and then diffused through this non-living material into the stump where it accelerated growth on the dark side, causing bending toward the lighted side. Boysen-Jensen also showed that he could intercept the substance by inserting a small piece of mica into the coleoptile. If the mica was inserted on the illuminated side, phototropic curvature occurred normally; if inserted on the dark side, very little or no bending occurred. Similar experiments were performed with geotropically stimulated coleoptiles. If the horizontally placed organs had mica inserted on the upper side of the tip, negative geotropism resulted as in normal coleoptiles; if the mica was inserted on the lower side, little or no bending occurred. The interpretation which Boysen-Jensen put upon the results of these experiments was that the stimulus originating in the tip was of a chemical nature rather than physical and that it acted in regulating growth. He thought there was an increased transmission of the growth-promoting substance on the dark side, a view which is still tenable. Due to the importance of the subject and the adverse views (Went and Thimann 37, p. 11) of the interpretations which Boysen-Jensen put upon the results of his experiments, it is deemed advisable to quote an entire paragraph from one of his early publications (3, p. 24): "Si je rappelle ces expériences, c'est parce qu'il me semble que mes expériences sur la transmission de l'irritation dans le coléoptile de l'avoine rendent vraisemblable que dans ce cas la transmission de l'irritation est de nature matérielle produite par des changements de concentration dans la pointe du coléoptile. En tous cas il me paraît qu'il faut laisser de côté l'hypothèse d'après laquelle la transmission d'irritation dans l'avoine serait dû à des causes physiques (changements de pression, etc.), ce qui est peut-être le cas pour le mimosa; en effet nous avons vu que l'irritation peut se transmettre à travers une incision pratiquée dans le coléoptile. Par contre diverses raisons font penser que la transmission de l'irritation est de nature chimique. Comme on s'en souvient, la condition due passage de la transmission à travers une incision était que les lèvres de la blessure fussent maintenues humides et serrées l'une contre l'autre, de façon à favoriser autant que possible une transmission de substance ou d'ions à travers l'incision. Autre raison: on n'a jamais pu constater de transmission de l'irritation à travers une incision lorsque les plantes d'expérience se trouvaient sous l'eau. L'eau doit être en état d'empêcher cette transmission, ce qui ne peut s'expliquer que dans l'hypothèse où la transmission de l'irritation serait dû à une migration de substance ou d'ions, qui se diffusent dans l'eau et ne peuvent plus agir." This shows that Boysen-Jensen actually realized that chemical substances were involved in tropic responses and thus the groundwork for the modern plant hormone concept was laid.

Paál (25, 26) during 1914 to 1918 confirmed Boysen-Jensen's results

and further showed that if an excised coleoptile tip was replaced on one side of the stump, growth was accelerated on that side, resulting in curvature. For this response no special stimulation of the tip was necessary, thus showing that the tip also in the dark was continually making the growth hormone. Paál also demonstrated that the stimulus passed through an interposed gelatinous membrane 0.1 mm. in thickness between the tip and the stump of the coleoptile. He concluded from his experiments that the transmission of the phototropic stimulus was brought about by means of a diffusible substance (26, p. 431).

Stark (34) in 1921 made the next big advance by investigating the transmission of phototropic, traumatotropic (33), and haptotropic stimuli. He expressed the sap from coleoptiles and mixed it with agar. Out of the agar plate, blocks were cut and placed unilaterally on decapitated coleoptiles. A substance drained out of the block into one side of the coleoptile, retarding growth and causing curvature.

Seubert (30) in 1925 extended the experiments of Stark by infiltrating agar with substances of both plant and animal sources—diastase, malt extract, saliva, etc.—demonstrating the existence of both accelerating and inhibiting substances.

Purdy (27) [1920–1921 while a fellow of the American-Scandinavian Foundation working in the plant physiology laboratory of the University of Copenhagen] repeated and confirmed the essential results of Boysen-Jensen. The work was concerned especially with the path of transmission of the stimulus. To show how nearly the early ideas and theories paralleled present day conclusions the following passage is quoted from Purdy's publication (27, p. 27): "Since the most probable theory at the present time is the migration one, let us attempt on this ground to interpret briefly the results of the foregoing experiments. To produce a curvature a difference must exist not between the front and back of each cell but between the front and back of the unilaterally illuminated tip as a whole. This condition may be brought about by an unequal concentration of one or more substances. Furthermore it may be presumed that this difference is maintained by transmission of the stimulus to the base of the coleoptile. Only under these circumstances is it possible to conceive of a phototropic or geotropic curvature taking place."

Söding (32) in 1925 showed that in decapitated coleoptiles the growth was diminished but increased again when the tip was replaced.

Cholodny (10, 11, 12) from 1924 to 1927 promoted the idea of displacement or unequal distribution of growth substances in phototropically or geotropically stimulated organs. The results of his experiments supported his theory that geotropic bending of shoots is due to redistribution of natural hormones in favor of the lower side of the organ. Growth being stimulated on that side caused negative geotropic curvature. Photo-

tropic curvature is due to migration of the substance to the dark side of the stem. Geotropic stimulation of roots caused the same unequal distribution of hormones as in shoots but the different direction of growth was due to the fact that hormones retard the rate of growth of roots. Cholodny's experiments supported the theories for both roots and shoots.

Went (35, 36) in 1928 demonstrated that it was not necessary to express the sap from the coleoptile to collect the hormone in agar; if allowed to stand with the cut surface on agar the substance would drain from the excised tip into the agar. The imbibed agar could then be used as in the experiments of Stark and Seubert. The method of Went proved useful in a number of ways and led to the biological test for quantitative determination of natural substances. Since the degree of curvature induced in coleoptile stumps varied with the concentration of substance in the agar blocks, it became possible to determine the comparative amounts of substance produced by different plants or parts of plants under varying conditions. The method has been adapted also for quantitative assay of activity of synthetic substances and hormones. Using this method Went (36) detected more growth substance on the dark side of a unilaterally lighted coleoptile tip than on the illuminated side.

Dolk (17) in 1929 found that an excised coleoptile tip placed in a horizontal position with the cut surface in contact with two agar blocks (above and below) yielded more hormone to the lower block than the upper. Also, in a cylindrical segment of a coleoptile, placed in a horizontal position and treated on the apical end with agar containing the hormone, the substance was transported to the basal end. When it was collected there in two blocks (upper and lower) of agar, the lower block yielded more hormone than the upper, but the total amount remained the same. The results of these experiments showed definitely that there was unequal displacement of the active substance in the coleoptile tissue under geotropic stimulation.

Boysen-Jensen (5) in 1933 made another important advance by proving that root tips produce growth-promoting substance and that when they are placed in a horizontal position more of the hormone is given off from the lower than the upper side of the excised tip.

For further reviews of the literature reference should be made to a survey of the field and citations in Boysen-Jensen's recent publication (6, 7) or reviews by Jost (21), or Schlenker (28).

The present paper is concerned with natural and induced tropic responses of plants. Three of the most effective synthetic growth substances were administered in various ways to intact plants, excised shoots, and plants with tops removed while oriented other than in their normal equilibrium position. Regardless of whether the substances were applied to the roots, the tips, or basal ends of excised shoots, the final

results were similar. There was some evidence that the synthetic growth-promoting substances were unequally distributed in the tissues when the shoots were placed in the horizontal position as reported for natural hormones. Low concentrations accelerated negative geotropic responses, while higher concentrations induced positive (toward the earth) bending. Plants left in the dark several days lost their capacity to right themselves when oriented in a horizontal position. They regained the power to respond to gravity when treated with solutions of the growth substances. Surface measurements of the rates of growth made during natural and induced tropic responses showed that growth occurred in all treated shoots regardless of their response to gravity. Time-lapse motion pictures were used to record and study the natural and induced responses while the plants were upright or oriented in a horizontal or an inverted position.

MATERIAL AND METHODS

Five species of plants were used in the experiments as follows: tomato (*Lycopersicon esculentum* Mill.), Klondike cosmos (*Cosmos sulfureus* Cav.), African marigold (*Tagetes erecta* L.), sunflower (*Helianthus debilis* Nutt.), and tobacco (*Nicotiana tabacum* L. var. Turkish). In general the tomato plants were the most satisfactory because they were uniform and easy to grow in large numbers.

Intact plants in pots were treated while upright, inverted, or in a horizontal position. Water solutions were applied to the soil while the plants were upright. After the solutions had gone into the soil the plants were oriented as desired.

When lanolin preparations were used, the intact plants were placed in a horizontal position and the substance was applied around the stem to a zone an inch wide at the tip, near the middle, or at the base of the stem.

Tips were removed from potted plants and water solutions of the substances were applied through glass tubes attached to the stump with rubber tubing. For treatment of horizontal stems the glass tubes were bent L-shaped so the solution could be kept in contact with the cut surface of the stump. U-shaped tubes were used for inverted plants and straight tubes for upright plants.

The majority of the experiments were conducted with severed leafy shoots four to six inches in length. The solutions were applied at the base through glass tubes when the shoots were horizontal or inverted. In many experiments the upright, horizontal, and inverted shoots were connected to the same reservoir containing the solution, as shown by Figure 1.

The growth substances, α-naphthaleneacetic acid, β-indoleacetic acid, and γ-indolebutyric acid were manufactured and supplied by Merck & Co. Inc., Rahway, New Jersey. The concentrations of the substances employed in the experiments are given under "Experimental Results."
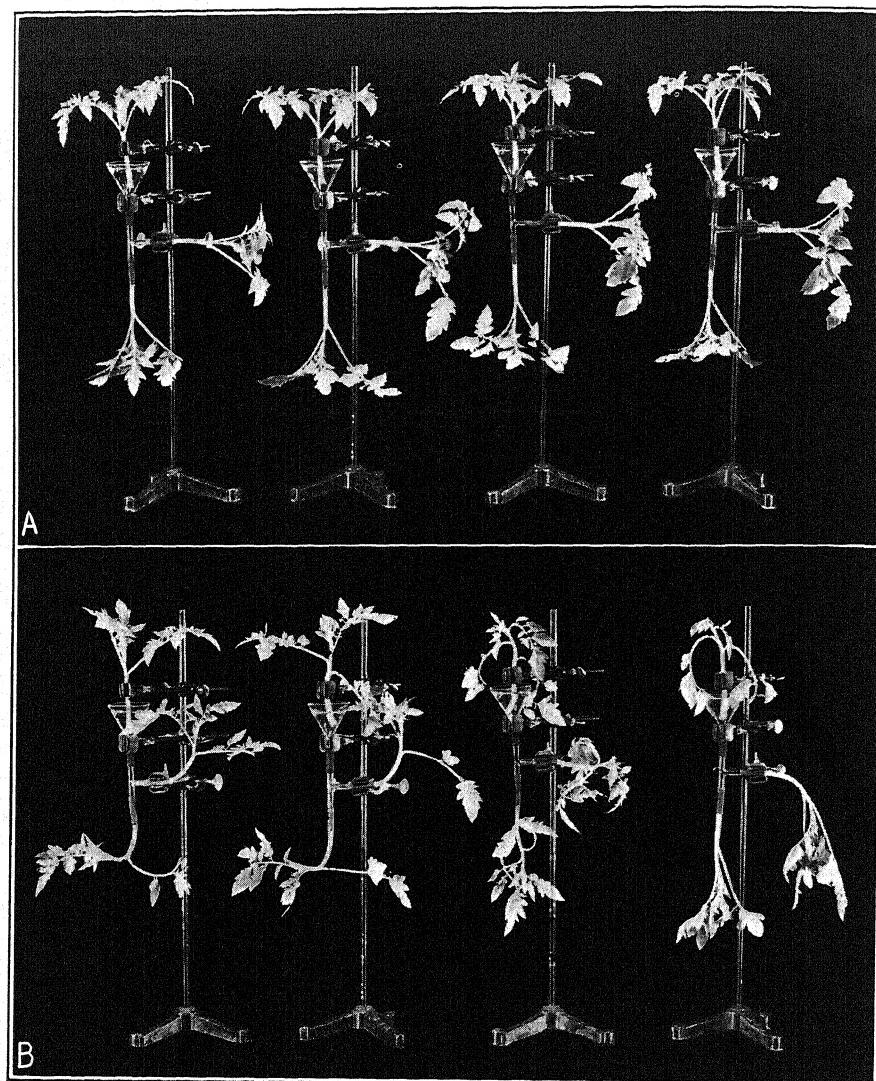
FIGURE 1. The effect of the relative position of the shoot with reference to gravity during treatment with water solutions of naphthaleneacetic acid. Concentrations are as follows (left to right): controls; 0.1 mg./l.; 1.0 mg./l.; 10.0 mg./l. A. The appearance of the shoots at the beginning of the experiment. B. The appearance of the plants 24 hours later. Note the epinasty of leaves of upright shoots and hyponasty of inverted shoots. Also note changing geotropism of horizontal stems with increasing concentration.

Motion pictures were made of plants while under treatment with automatic cinema equipment. Exposures were made at the rate of about six per minute. When shown on the screen, the speed was approximately 160 times normal. In some cases the pictures were taken over a 24-hour period. The best pictures were taken at the rate of six per minute over a five-hour period.

EXPERIMENTAL RESULTS

Plants were treated with the substances while the shoots were in a horizontal position, in the normal upright position, or while inverted (40). A method for treating excised shoots is illustrated in Figure 1. Due to the variation in the nature of the response according to the position of the shoots it is necessary to describe the results separately for each group. Though five different species were used in the experiments, the results given apply to tomato unless otherwise stated.

Horizontal stems. Four different methods were employed for studying tropic responses of stems placed in a horizontal position as follows: Water solutions of growth substances applied to the basal end of excised shoots; water solutions applied to soil in which the roots of intact plants were growing; water solutions applied to top of the stump after an inch or more of the tip was removed; and lanolin preparations applied around a short portion of the stem at various places from tip to base.

Excised shoots of tomato, cosmos, sunflower, tobacco, and marigold were treated with water solutions as shown in Figure 1. The three chemicals, naphthaleneacetic acid, indolebutyric acid, and indoleacetic acid, induced similar responses though there was considerable variation in concentrations required to bring about a given degree of bending. For the two indole compounds a range of concentrations from 0.1 mg. to 100.0 mg. per liter of water induced any of the responses described. A more narrow range (0.1 to 10 mg./l.) served for naphthaleneacetic acid. At the lower concentrations treated stems showed negative geotropic response similar to water controls, though the substance appeared to accelerate the rate of growth. The higher concentrations induced positive geotropism. At some point between the two extremes there was a concentration that caused the stem to continue growing in a horizontal position. The exact concentration requirements varied somewhat with the condition of the plants. Light conditions prior to treatment were especially effective in modifying the capacity of the plant to respond (20, 39). The pictures of horizontal stems in Figure 1 illustrate the three types of response induced with naphthaleneacetic acid.

A total of more than 1000 stems have been treated in the course of these experiments to study induced tropisms under a variety of conditions. Typical sets of results are shown in Tables I and II.

Geotropic bending is assumed to be due to variation in the rate of growth on upper and lower sides of the stem. In order to determine the relative growth rates the two sides were stamped with a marker having

TABLE I

COMPARATIVE GROWTH RATES ON UPPER AND LOWER SIDE OF STEMS WHEN SEVERED TOMATO SHOOTS WERE PLACED IN A HORIZONTAL POSITION AND TREATED AT BASE WITH WATER SOLUTIONS OF INDOLEACETIC ACID. NEGATIVE BENDING (AWAY FROM THE EARTH) INDICATED BY THE MINUS (−) SIGN; STRAIGHT GROWTH BY ZERO (o) AND POSITIVE BENDING BY THE PLUS (+) SIGN. MEASUREMENTS WERE MADE BY MEANS OF SURFACE MARKINGS. READINGS WERE TAKEN 18 HOURS AFTER THE EXPERIMENTS STARTED

Concentration, mg./l.	Degree of curvature	Percentage increase on the two sides	
		Upper	Lower
Control	− 90	6.1	18.6
"	− 90	4.5	22.4
"	− 80	6.8	19.5
"	− 90	4.8	14.6
"	− 90	4.1	15.9
Average	− 88	5.3	18.2
1.0	− 90	7.5	21.6
1.0	−100	8.5	23.1
1.0	− 50	16.3	25.3
3.0	− 10	17.3	22.7
Average	− 63	12.4	23.2
5.0	o	25.1	24.6
5.0	o	18.6	18.4
5.0	− 5	17.1	21.8
5.0	o	15.1	17.8
7.0	o	28.2	26.7
Average	− 1	20.8	21.9
10.0	+ 45	34.6	18.2
50.0	+ 60	15.5	10.2
50.0	+ 60	29.6	29.1
100.0	+ 60	20.0	13.8
Average	+ 56	24.9	17.8

three millimeter spaces between the lines, and then measurements were made at various intervals after treatments. Considerable growth occurred in four hours. A pronounced response occurred in 24 hours. The figures showing the results of the measurements (Tables I and II) indicate that growth occurred on both sides of geotropically stimulated stems but the convex side elongated more than the concave side. Stems that remained in a horizontal position grew at approximately the same rate on all sides of the stem. In all cases where curvatures occurred the total growth of both sides of the controls was less than that of treated stems. Where straight, horizontal growth occurred, the average growth of both sides

was greater than the average growth of both sides of the controls. Treated shoots which showed negative geotropism grew considerably more on the upper side than corresponding controls. Also, shoots that were induced

TABLE II

COMPARATIVE GROWTH RATES ON UPPER AND LOWER SIDE OF STEMS WHEN SEVERED TOMATO SHOOTS WERE PLACED IN A HORIZONTAL POSITION AND TREATED AT BASE WITH WATER SOLUTIONS OF NAPHTHALENEACETIC ACID. NEGATIVE BENDING (AWAY FROM THE EARTH) INDICATED BY THE MINUS (−) SIGN; STRAIGHT GROWTH BY ZERO (o) AND POSITIVE BENDING BY THE PLUS (+) SIGN. MEASUREMENTS WERE MADE BY MEANS OF SURFACE MARKINGS. READINGS WERE TAKEN 18 HOURS AFTER THE EXPERIMENTS STARTED

Concentration, mg./l.		Degree of curvature	Percentage increase on the two sides	
			Upper	Lower
Control		− 80	o	13.9
Control		− 90	o	17.6
	Average	− 85	o	15.8
0.1		− 50	4.3	15.3
0.1		− 80	8.3	15.0
	Average	− 65	6.3	15.2
0.5		− 50	9.1	21.3
0.5		− 60	6.6	21.8
	Average	− 55	7.9	21.6
1.0		o	15.5	13.7
1.0		o	16.3	13.7
	Average	o	15.9	13.7
5.0		+ 45	23.4	14.8
5.0		+ 30	13.0	6.6
	Average	+ 38	18.2	10.7
10.0		+ 85	17.1	9.3
10.0		+ 70	23.6	13.9
	Average	+ 78	20.4	11.6

to bend toward the earth showed more growth on the concave side than the controls.

Stems placed in a horizontal position and left until they showed slight negative geotropism were then turned through 180° so that the upper side of the stem became the lower side. The final bending of these plants was considerably less than that of controls which were not turned. The stems that were turned at intervals of two and four hours for a period of eight hours, and finally left without further disturbance, showed much less negative geotropism than normal plants. Measurements by means of surface markings indicated that both sides of the stem had been stimu-

lated (Tables V and VI). The convex side of controls which had not been turned after being placed in a horizontal position showed practically no change in 24 hours. Plants that were turned at intervals for eight hours and then left in a horizontal position showed negative geotropism near the tips within 24 hours. Eventually the older parts also showed considerable negative bending. Plants that were turned daily for four days showed as much as 77.8 per cent increase in length at the most active nine-millimeter region. The most active region was usually within 10 centimeters of the growing tip. The figures in Tables V and VI show that both sides continued to grow though repeatedly turned for geotropic stimulation.

Plants placed in a horizontal position and then treated along the upper side of the stem with lanolin preparations of growth substances were induced to bend downward through a 90° arc (39). However, plants that were allowed to make a pronounced negative geotropic response before the lanolin preparations were applied to the upper side of the stem, returned only to the horizontal position. Failure to bend downward as in the first case was due to growth on both sides of the stem. The lower side of the geotropically stimulated stem continued to grow after the upper (concave) side was treated. Measurements by means of surface markings showed that the total growth in 22 hours was approximately the same on both sides of the stem, though only the upper side had been treated with the lanolin preparation.

Intact potted plants with substances added to the soil responded as described for leafy shoots but higher concentrations were required. A range of 1 to 20 mg. of naphthaleneacetic and indoleacetic acid was used per pot. As much as 50 mg. of indolebutyric acid were used. The substance was dissolved in 50 cc. of water and the solution was poured over the soil (20). As soon as the liquid went into the soil the plants were placed so that the stems were in a horizontal position. Responses were apparent within one hour after the plants were treated. As with excised shoots, intact plants showed movement earlier in treated lots than in the controls. This fact was easily shown with time-lapse motion pictures taken at the rate of six exposures per minute.

Another experiment involving potted plants with tops removed was planned to determine the effect of substances applied at the apical end of the stump. Water solutions were applied to the cut surface through glass tubes coupled to the stem with rubber tubing. Here again the responses were approximately the same as where the substances were applied to the basal end of excised shoots or to the root system of intact plants. It was necessary, however, to use higher concentrations to induce positive geotropism than were employed for the excised leafy shoots (Fig. 2). Naphthaleneacetic acid was more effective for inducing positive geotropism than indoleacetic acid and the latter was more effective than indolebutyric
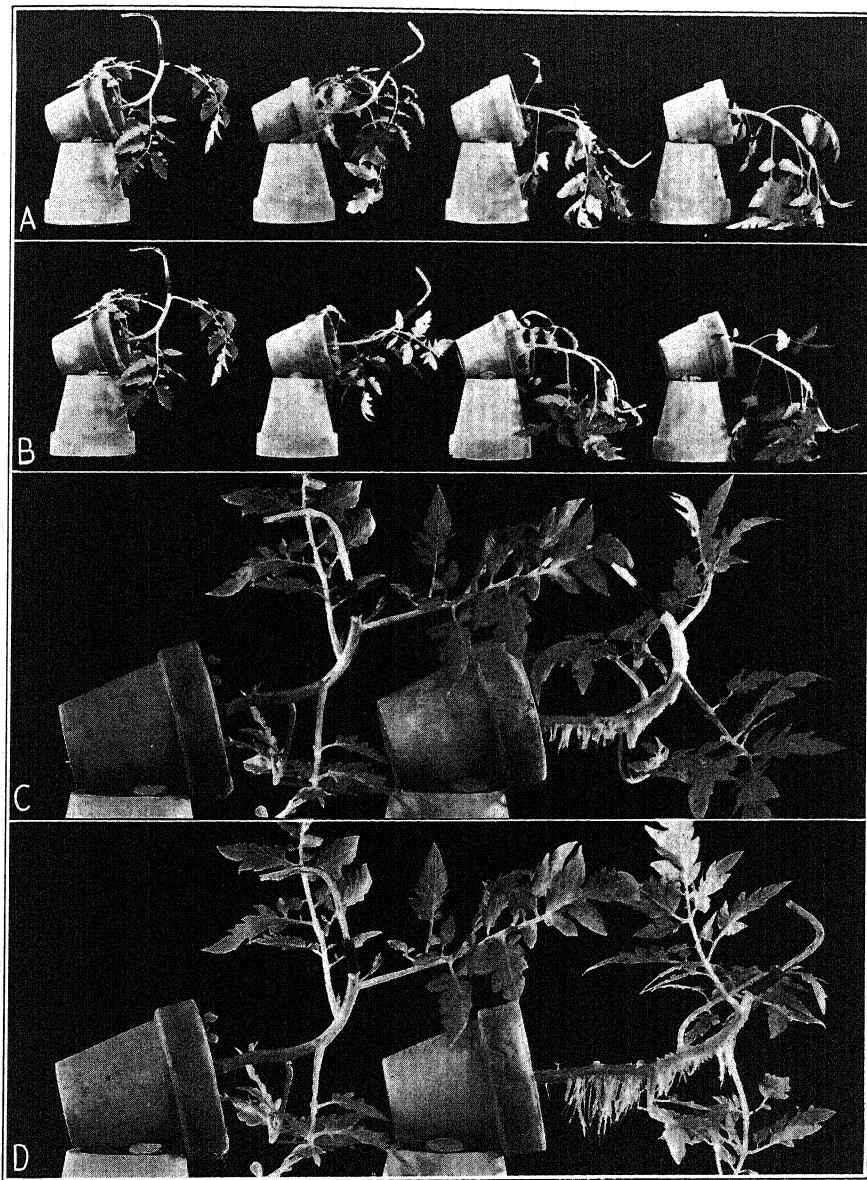
FIGURE 2. Tomato plants with tips removed and treated at top with water solutions by means of glass tubes while horizontal. A. Naphthaleneacetic acid series (left to right): control; 25 mg./l.; 50 mg./l.; 300 mg./l. B. Indoleacetic acid series (left to right): control; 50 mg./l.; 100 mg./l.; 300 mg./l. Both A and B photographed after 24 hours. C. Control (left) and treated with naphthaleneacetic acid 50 mg./l. D. Control (left) and treated with indoleacetic acid 300 mg./l. Both C and D photographed after 12 days.

acid. To prevent negative geotropism and induce straight growth or slight positive geotropism, the following concentrations were employed: naphthaleneacetic acid, 5 to 10 mg./l.; indoleacetic acid, 50 to 100 mg./l.; indolebutyric acid, 100 to 200 mg./l.

Intact plants were placed in a horizontal position and then treated at various places around the stem with lanolin preparations of the three substances. The response varied with the distance of the treated region from the tip. If a zone of one inch around the stem near the tip was treated with a 1.0 per cent lanolin preparation of indoleacetic acid or 0.5 per cent naphthaleneacetic acid, positive geotropism was induced. Tomato stems ten inches in length treated near the middle showed negative geotropism at the tip and positive near the center of the stem. Observed as a whole the stem had an S-shaped appearance. Various degrees of bending were obtained by shifting the treatment toward the base (Fig. 3).

The responses induced by treatment around the stem are in contrast with those brought about by unilateral treatment. When one side of the stem is treated, negative bending occurs due to the local effect. From the results obtained it appears that when treated around the stem, the substances were taken in on all sides. It was then distributed in both directions to distant places, most of it going to the lower side of the horizontal stems. With respect to the possible inhibiting action of growth substance applied below the principal growing region, our results support Snow's more recent growth acceleration hypothesis (31) rather than the inhibiting hypothesis of Le Fanu (24).

Upright versus inverted stems. Upright severed shoots treated at the base with a water solution of the growth substances show epinasty of leaves over a wide range of concentrations (discussed more fully under another heading). In contrast with this response, inverted shoots, treated at the base with high concentrations of the substances, show no epinasty and no geotropism of the stem. Where low concentrations were used, the stems made negative geotropic responses and epinasty was discernible on the righted portions. It is assumed that when inverted stems were treated with high concentrations the substance was more or less uniformly distributed through the tissue. Therefore, growth being generally accelerated, no bending occurred. It was noted that in controls where the stem pointed directly toward the center of the earth, the rate of geotropic response was slow as contrasted with stems placed in a horizontal position. Where high concentrations were used, growth appeared to be accelerated nearly equally on all sides (Tables I and II). Data on root formation (appearing under another heading) also confirm this view. Measurements of growth were made by stamping India ink uniformly along opposite sides of the stem for a distance of 72 mm. beginning with the tip. The marks were three millimeters apart. A vernier caliper was used to determine the change in dis-
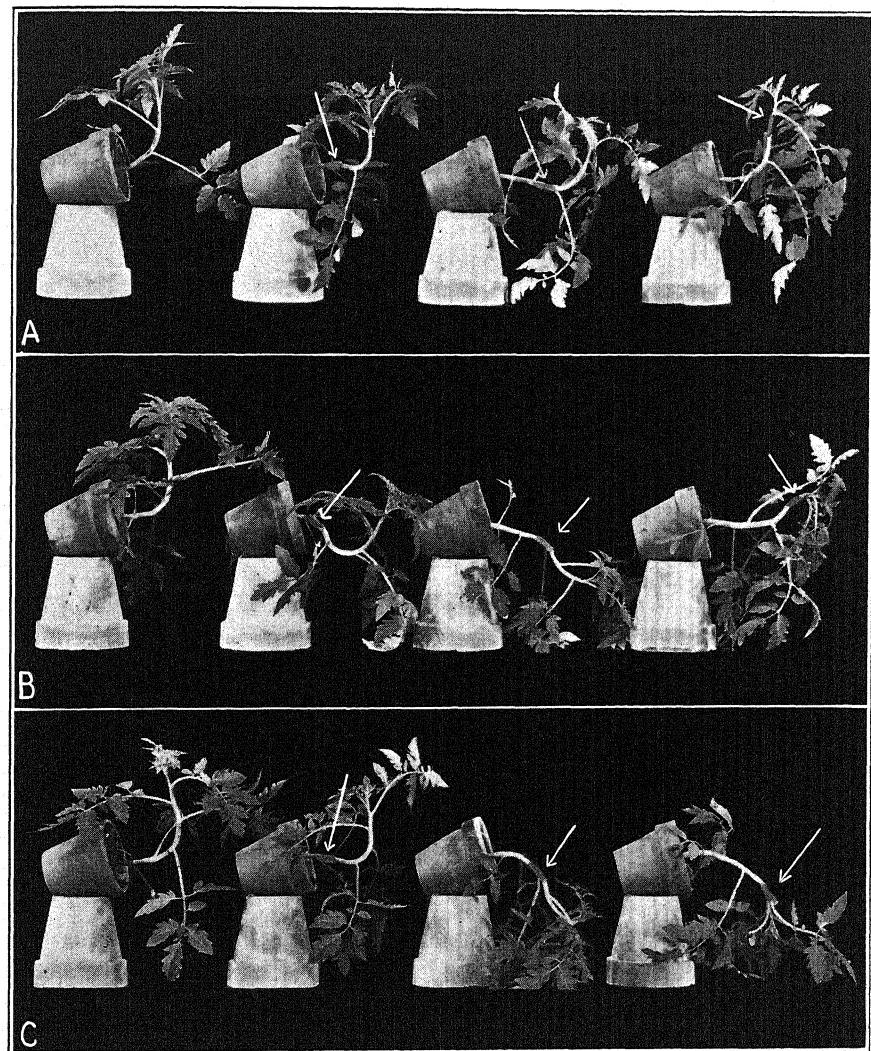
FIGURE 3. Tomato plants treated at various places (see arrows) around the stem with lanolin preparations of the substances while horizontal and photographed after 24 hours. Control on left in each case. A. Indolebutyric acid 1.0 per cent. B. Indoleacetic acid 0.5 per cent. C. Naphthaleneacetic acid 0.5 per cent.

tance between the marks occurring during treatment. Tables III and IV show the percentage increase in growth occurring when stems were upright and inverted during treatment. Figure 1 shows the appearance of the upright and inverted shoots at the beginning of the experiment and 17 hours later.

TABLE III

COMPARATIVE GROWTH RATES ON TWO SIDES OF THE STEM OF UPRIGHT AND INVERTED TOMATO SHOOTS DURING TREATMENT AT THE BASE WITH WATER SOLUTIONS OF NAPHTHALENEACETIC ACID. WHERE CURVATURES OCCURRED THE CONCAVE SIDE IS MARKED "LEFT." MEASUREMENTS WERE TAKEN 21 HOURS AFTER THE EXPERIMENT STARTED

Concentration, mg./l.	Relative position of shoot	Bending in degrees from vertical	% increase in the two sides marked with India ink	
			Left side (concave)	Right side (convex)
Control	Upright	0	7.1	10.1
Control	"	0	6.1	5.4
Control	"	0	8.2	4.9
Average		0	7.1	6.8
0.1	"	0	10.7	13.3
0.1	"	0	8.8	7.9
Average		0	9.7	10.6
1.0	"	0	22.3	21.1
1.0	"	40	16.4	18.5
Average		20	19.3	19.8
10.0	"	60	19.2	34.6
10.0	"	0	19.9	22.5
Average		30	19.5	28.5
Control	Inverted	130	3.3	15.3
Control	"	140	1.9	20.8
Control	"	100	3.2	23.3
Average		123	2.8	19.8
0.1	"	140	1.8	19.7
0.1	"	130	4.0	19.7
Average		135	2.9	19.7
1.0	"	100	8.6	23.7
1.0	"	140	3.8	21.1
Average		120	6.2	22.4
10.0	"	10	16.8	25.1
10.0	"	20	16.4	24.7
Average		15	16.6	24.9

Epinasty and hyponasty of leaves. The daytime equilibrium position of leaf petioles of small tomato plants (5 to 10 inches in height) was usually above the horizontal. The angle formed by the upper side of the petiole and the stem ranged from 15° to 90°. The smallest angles were nearest the tip of the plant. With aging, the leaves gradually moved downward (epinasty) often going beyond the 90° mark.

TABLE IV

COMPARATIVE GROWTH RATE ON TWO SIDES OF STEM OF UPRIGHT AND INVERTED TOMATO SHOOTS DURING TREATMENT WITH TWO SUBSTANCES. EXPERIMENT SIMILAR TO ONE OF TABLE III BUT DIFFERENT LOT OF PLANTS USED

Concentration, mg./l.		Position of shoot	Bending in degrees from vertical	% increase in stems during treatment	
				Left side (concave)	Right side (convex)
Control		Upright	0	2.5	2.1
Control		Upright	0	5.6	4.0
Control		Upright	0	4.0	5.2
	Average		0	4.0	3.8
Naphthaleneacetic	0.1	Upright	0	4.9	8.1
	0.1	Upright	30	7.5	10.5
	0.1	Upright	15	8.6	9.1
	Average		15	7.0	9.2
Naphthaleneacetic	1.0	Upright	10	11.6	14.1
	1.0	Upright	0	14.8	8.3
	1.0	Upright	35	13.2	19.4
	Average		15	13.2	13.9
Naphthaleneacetic	10.0	Upright	10	5.7	8.3
	10.0	Upright	0	14.4	13.0
	10.0	Upright	0	21.9	20.9
	Average		3	14.0	14.1
Control		Inverted	150	2.7	24.0
Control		Inverted	35	5.2	13.0
	Average		93	3.9	18.5
Naphthaleneacetic	0.1	Inverted	20	8.1	13.0
	0.1	Inverted	90	3.2	16.2
	0.1	Inverted	20	10.8	11.6
	Average		43	7.4	13.6
Naphthaleneacetic	1.0	Inverted	0	11.7	10.8
	1.0	Inverted	0	15.1	10.3
	1.0	Inverted	0	14.1	17.1
	Average		0	13.6	12.7
Control		Inverted	120	3.0	13.8
Control		Inverted	135	2.7	15.7
Control		Inverted	115	—0.7	13.0
	Average		123	1.6	14.2
Indoleacetic	1.0	Inverted	60	4.0	11.0
	1.0	Inverted	100	7.2	16.2
	Average		80	5.6	13.6
Indoleacetic	10.0	Inverted	30	9.1	12.1
	10.0	Inverted	35	12.7	15.9
	Average		33	10.9	14.0
Indoleacetic	50.0	Inverted	30	17.2	17.8
	50.0	Inverted	20	15.9	15.6
	50.0	Inverted	10	9.8	11.3
	Average		20	14.3	14.9

At night, young leaves of many plants (tomato, cosmos, marigold, etc.) move upward (hyponasty) until they nearly parallel the stem. The middle-aged leaves show less hyponasty at night and the older leaves show practically none.

Epinasty of upright plants can be induced in a number of ways. Exposure of the plants to an atmosphere containing traces of ethylene, acetylene, propylene, or carbon monoxide gas induces epinasty, when the plants are in an upright position (13, 14). Similarly, many synthetic chemical compounds, commonly called growth substances, induce epinasty of leaves when applied with water to soil in which plants are growing (20). Severed leafy shoots placed upright with the basal ends in water solutions of the growth substances or water through which gas is bubbled promptly show epinasty of leaves.

Inverting the plants or otherwise orienting them away from their normal equilibrium position with reference to gravity changed their capacity to respond to treatment with growth substances and ethylene gas (14). When high concentrations of the substances were applied to the basal ends of severed, inverted shoots the stems remained straight and leaves showed hyponasty (Fig. 1). The petioles pressed down against the stem and the blades curled up. The degree of hyponasty increased with the concentration of the substances.

When low concentrations were used the young part of the stem made a negative geotropic response and leaves on the righted parts exhibited epinasty.

A range of concentrations from 0.1 to 300.0 mg. per liter of water was tested. There was considerable variation in effectiveness of the substances. The responses described for severed shoots could be obtained with the three chemicals used in concentrations as follows:

	Low	Medium	High
Naphthaleneacetic acid	0.1 mg./l.	1.0 mg./l.	10 mg./l.
Indoleacetic acid	0.5 "	5.0 "	100 "
Indolebutyric acid	0.5 "	5.0 "	200 "

The results given for experiments with severed shoots hold also for potted plants though the concentrations required when substances are added to the soil are higher than required when water solutions are applied to bases of leafy shoots. Figure 4 shows plants responding when oriented in three different positions. As with the leafy cuttings, the concentrations required varied with the substances and size of the pot in which the intact plant was growing. The various responses with potted plants could be obtained when the following amounts of chemicals were added to the soil in 4-inch pots:

	Low	Medium	High
Naphthaleneacetic acid	1 mg./pot	5 mg./pot	10 mg./pot
Indoleacetic acid	5 "	10 "	20 "
Indolebutyric acid	5 "	20 "	40 "

As a rule the substances were dissolved in 50 cc. of water which was added at one time to the soil of the 4-inch pot. There was evidence that
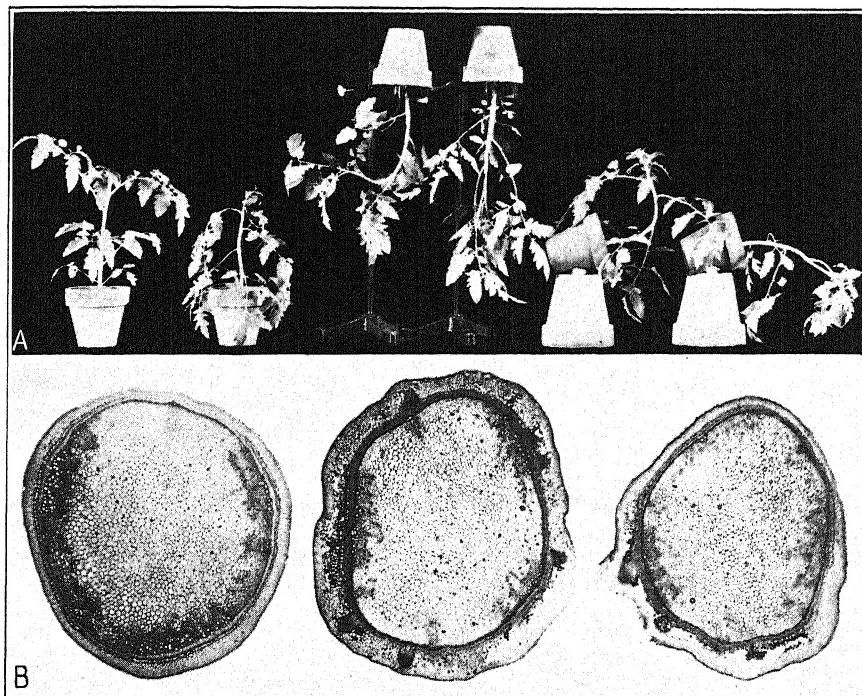
FIGURE 4. A. Tomato plants in three positions after treatment with 20 mg. of indoleacetic acid per pot applied to the soil in 50 cc. of water; control on left in each group. Photographed after 24 hours. B. Microscopic cross sections of stems approximately 10 inches above soil taken seven days after treatment of soil with 10 mg. naphthaleneacetic acid. Left, upright control; middle, inverted treated; right, horizontal treated. Note root primordia and thick cortex all around the inverted and on the lower side of horizontal stem in contrast with the control.

the responses were not due to the total amount of the solution added but instead to the concentration of the substance in the solution.

The effect of dark on the response of plants. Plants which were kept in a dark room three to five days prior to treatment with growth substance were more sensitive than similar plants taken directly from greenhouses. Tomato plants kept in the dark for five days lost their capacity to respond

to the force of gravitation when placed in a horizontal position. They were, however, induced to respond when the growth substances were applied either as lanolin preparations or as water solutions (39). Concentrations which normally induce negative bending of horizontal stems produced positive bending of plants which were kept in the dark for two to five days, showing that darkness increased the sensitivity. The temperature at which plants were stored in the dark rooms varied the rate at which they lost their natural hormone. Storage at 50° F. enabled the plants to maintain their capacity to respond much longer than those stored at 80° F.

As this paper was being prepared there appeared an article by Botjes (1) showing that our earlier work concerning the effect of darkness on plants was successfully repeated and extended in an interesting way. After the plants lost their power to respond to gravity, Botjes placed them in a horizontal position for 24 hours and then turned them around 90° and left them for another 24 hours. At this time a lanolin preparation of indoleacetic acid was applied around the stem. Bending occurred away from the side which was lowest when the plant was first placed in a horizontal position.

Epinasty induced by artificial orientation of the plants. When plants are placed in a horizontal position the stems are brought into equilibrium in respect to the earth by growing faster on the lower side and thereby making a negative geotropic response. After a plant has made this type of response it shows a peculiar epinasty of leaves after being placed again in an upright position. Figure 5 shows three tomato plants, one in a normal position, one showing negative geotropism, and the other a plant which was placed in a horizontal position, left for 24 hours, and then again placed in an upright position. The photograph was taken 18 hours later. Note that leaves show epinastic response as if they had been subjected to an atmosphere containing gas or treated with growth substances. It was not clearly understood how placing the plants as described above could bring about this type of response. It may be assumed that natural hormones have in some way been shifted to the upper side of the petiole. However, in no case was the plant placed so that the upper side of the leaf faced downward. White, swollen patches (proliferations) developed within three days along the stems, thus indicating that there was an abnormal distribution or increased production of natural hormones as the shoots resumed their normal equilibrium position. (Tables V and VI.)

Root formation and proliferations on treated plants. Intact tomato plants growing in 4-inch pots were treated by applying the substances to the soil and then placing the plants in various positions to be left for several days. The most striking response was the swelling of the tissue, proliferations, and the production of roots. Upright or inverted plants showed that
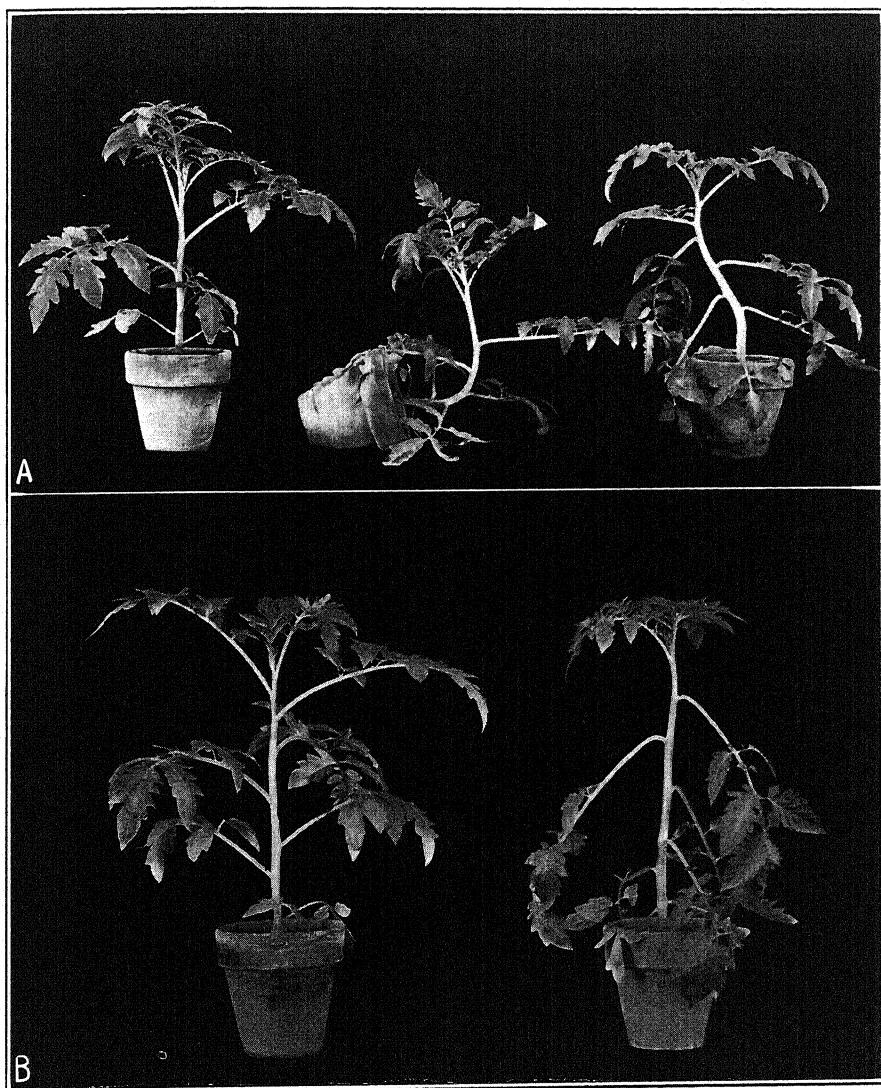
FIGURE 5. Tomato plants showing epinasty of leaves induced on geotropically stimulated plants without the application of growth substances. A. Left, control; middle, response after being placed in horizontal position; right, plant was placed in horizontal position and left 24 hours at which time it appeared like plant in middle; the pot was then righted and as the stem straightened up epinasty developed. B. Left, control; right, a rapidly growing plant treated as described for "A, right." Photographed after 24 hours.

TABLE V

COMPARATIVE GROWTH RATES ON THE UPPER AND LOWER SIDES OF STEMS OF UNTREATED
PLANTS PLACED IN A HORIZONTAL POSITION AND TURNED 180° AT INTERVALS FOR EIGHT
HOURS, THEN LEFT THE REMAINDER OF A 24-HOUR PERIOD WITHOUT FURTHER TURNING.
AFTER THE FIRST READINGS THE PLANTS WERE ALL TURNED WITH THE CONCAVE SIDE
DOWN AND LEFT FOR 24 HOURS WHEN THE SECOND READINGS WERE TAKEN

Intervals at which plants were turned (180°) during first 24 hours	Number of times turned in 24 hours	Angle in degrees after 24 hours	Measurements at two different times to show increase or decrease from the original length (63 mm.) of the measured zones. Figures show average of 3 stems			
			Upper side, % change		Lower side, % change	
			After 24 hours	After turned 180° and left for 24 hours	After 24 hours	After turned 180° and left for 24 hours
Control	0	86.6	−0.37	25.8	8.87	8.9
6 hours	1	40	6.33	27.8	13.01	13.9
4 hours	2	38.3	3.44	23.8	10.15	8.77
2 hours	4	40	3.85	22.3	9.47	8.5

the growth substance had affected the entire stem whereas the horizontal
plants were affected mostly on the lower side. This fact is illustrated by
Figure 4, showing intact plants and cross sections of inverted and hori-
zontal stems. The picture of the cross section of a treated, horizontal stem
indicates that the substance applied to the soil was unequally distributed
in the stem, the lower side receiving enough to cause abnormal growth in
the cortex and induce adventitious roots. Similar swelling and induction
of roots occurred all around the stems of upright or inverted plants which
had been given the soil treatment (20). The equal distribution of root
primordia and the equal growth of the cortex in vertically placed stems
are to be compared with the unequal distribution in horizontally placed
stems. For root induction in stems of intact plants the following range of

TABLE VI

INCREASE IN LENGTH OF A MARKED ZONE NEAR THE TIP OF PLANTS TURNED 180° AT IN-
TERVALS FOR FOUR DAYS. THE SAME PLANTS WERE MEASURED THE FIRST TWO DAYS FOR
DATA GIVEN IN TABLE V. THE DATA HERE GIVEN SHOW THE MOST ACTIVE
REGION WHICH WAS ORIGINALLY 9 MM. IN LENGTH

Treatment as shown in Table V	Upper side (concave)		Lower side (convex)	
	Mm.	% increase	Mm.	% increase
Control	14.0	55.6	15.7	74.4
Control	14.4	60.0	15.8	75.6
Control	13.0	44.4	14.0	55.6
Average	13.8	53.3	15.2	68.5
6 hour plant	15.0	66.7	15.7	77.4
6 hour plant	15.0	66.7	16.0	77.8
6 hour plant	14.8	64.5	14.8	64.5
Average	14.9	65.9	15.5	72.2
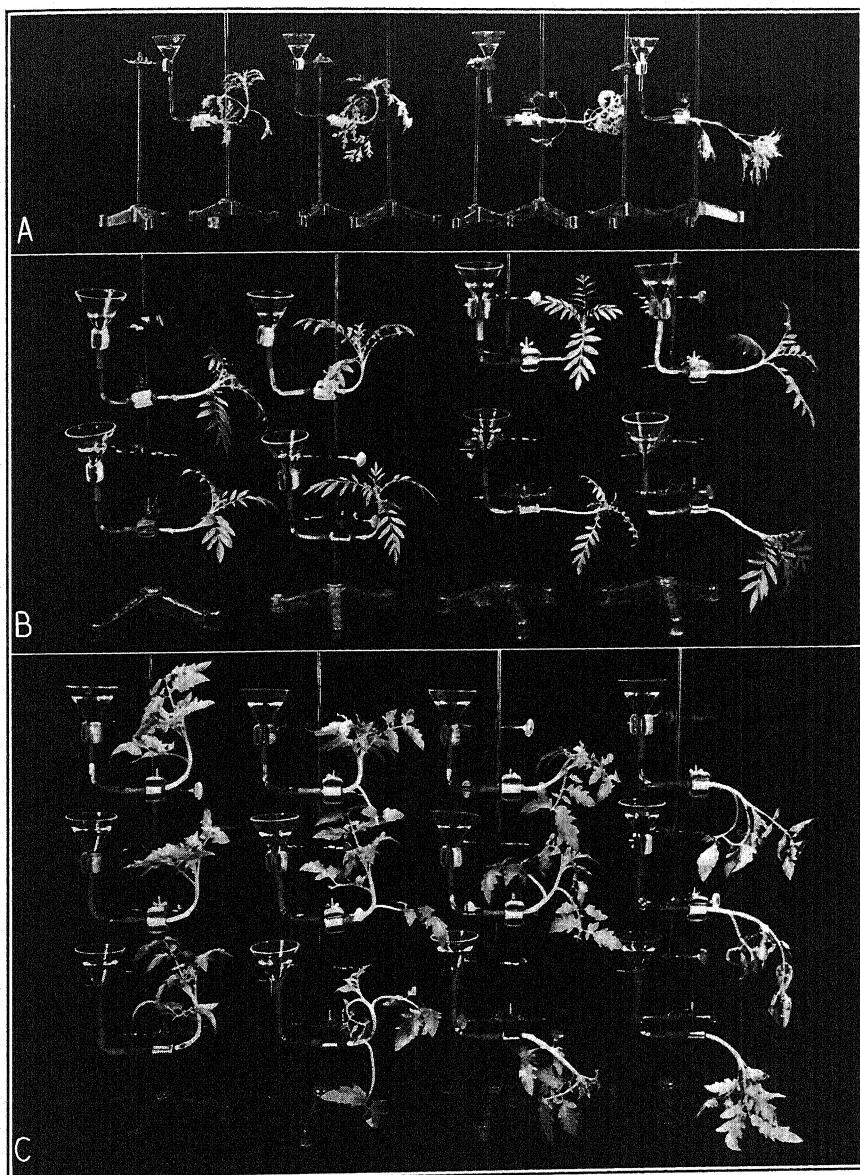
FIGURE 6. The response of horizontal, excised shoots of three species to various concentrations of the substances. A. Cosmos treated with indolebutyric acid: control; 10 mg./l.; 50 mg./l.; 100 mg./l. B. Marigold treated with indolebutyric acid (above) and naphthaleneacetic acid (below). Above (left to right): control; 1.0 mg./l.; 10.0 mg./l.; 100 mg./l. Below (left to right): control; 0.1 mg./l.; 1.0 mg./l.; 10 mg./l. C. Tomato treated with indolebutyric acid (above), indoleacetic acid (middle), and naphthaleneacetic acid (below). Concentration was same for all substances. Left to right: control; 0.5 mg./l.; 5.0 mg./l.; 100 mg./l.
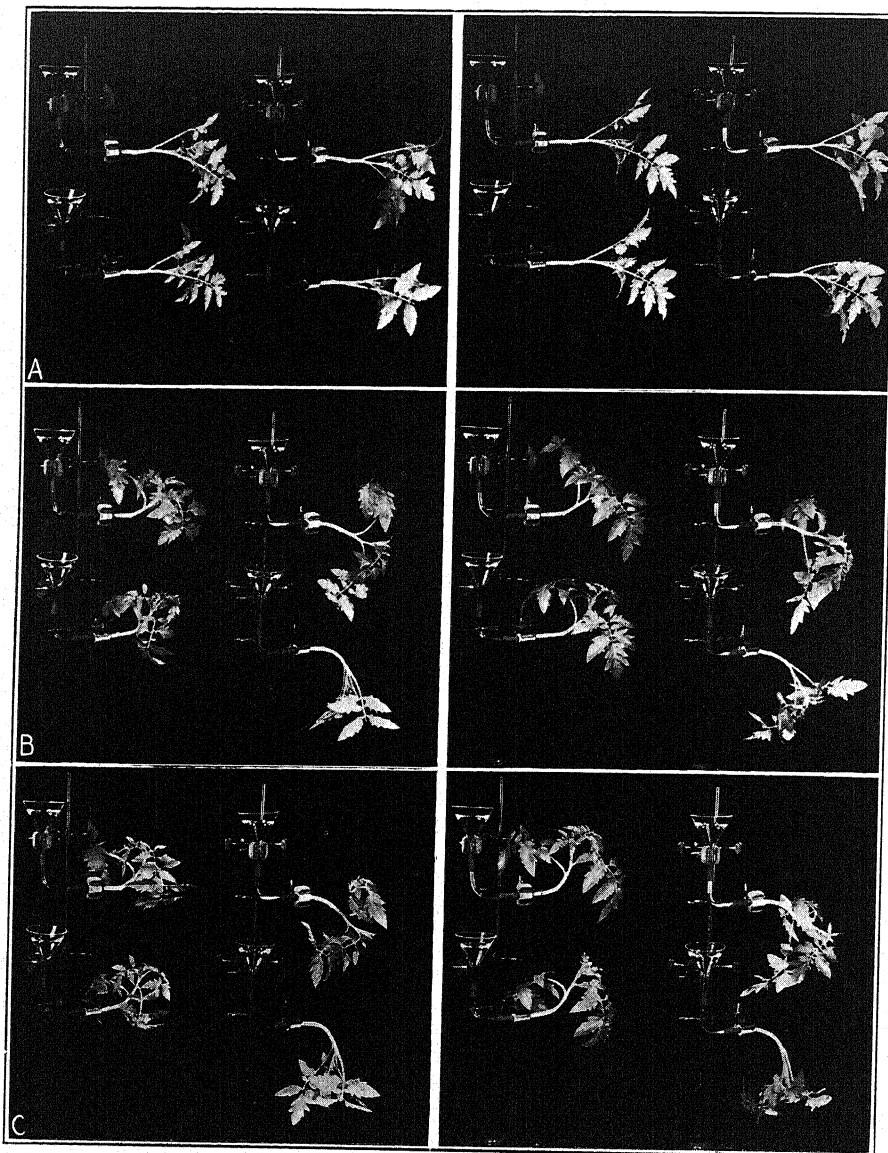
FIGURE 7. The response of tomato shoots (in replicate) to a range of concentrations of naphthaleneacetic acid, covering low (0.1 mg./l.); medium (1.0 mg./l.); high (10 mg./l.) concentrations for this substance. Photographs were taken (A) at the start; (B) after two and one-half hours; and (C) after five hours. The treatments were as follows: upper left, control; lower left, low concentration; upper right, medium concentration; lower right, high concentration. Both sets were used to make motion pictures, and lights were used only when exposures were made.

concentrations were found satisfactory for the three chemicals: naphthaleneacetic acid, 5 to 10 mg./pot; indoleacetic acid, 10 to 20 mg./pot; indolebutyric acid, 20 to 40 mg./pot.

Figure 6 shows comparative effectiveness of three different substances and the response of three different species. Figure 7 shows how a set of results can be duplicated.

DISCUSSION

This paper concerns particularly the systemic distribution and unequal redistribution of substances naturally occurring in plants or synthetic substances which are artificially applied while the plants are oriented other than their natural equilibrium position. The substances were applied at distal parts of stems and were then transported by the tissue and distributed according to the orientation of the shoots with reference to gravity. This method is in contrast to those previously reported showing that local responses could be induced by the application of lanolin preparations of growth substances. The present method of application permits the tissue to handle the synthetic substances as if they were natural hormones produced by the plant. As a result the responses are systemic rather than local.

Several unusual responses were described under the heading of "Experimental Results." A few additional remarks may be helpful to interpret results.

Negative geotropism of stems placed in a horizontal position is a well known response (22). Either the upper side does not grow or grows less than the lower side and therefore the stem bends away from the earth. But what could make a stem bend toward the earth as is the case with roots? The explanation for roots is simple enough. Natural hormones or synthetic substances retard growth of roots. Therefore, unequal distribution of substances in favor of the lower side accounts for positive geotropism of roots as well as negative geotropism of stems.

When positive geotropism of stems was first observed in the present experiments, after treating the basal end of a shoot with a high concentration of a growth substance, it was thought that growth on the lower side had been greatly retarded or perhaps stopped due to high concentration However, measurements of the rate of growth by means of surface marks on the two sides did not support that hypothesis. The data in Tables I and II indicate that growth was accelerated on both sides but the rate was greater on the upper side The assumption is that as the substance was distributed through the horizontal stem the upper side received near the optimum concentration for maximum growth. Whether the lower side received more or less than the optimum cannot be determined by surface measurements. In order to clarify these points it will be necessary to make

quantitative determinations of the amount of substance on both sides of the stem. When such information is at hand we shall understand whether or not the normal mechanism for redistribution has been altered. One has also to find an explanation for the horizontal stem that continues to grow straight after treatment with medium concentrations.

In the case of horizontal stems which curved only slightly or not at all there was a rapid growth rate on both the upper and lower sides. An assumption might be made that though most of the substance accumulated on the lower side, the concentration was still within the range which induces pronounced growth. At the same time the upper side might be assumed to have a concentration within the same range but near the lower limits. If the figures just cited are compared with those for upright shoots treated with the same concentration (Table III), it can be seen that the rates of growth are approximately the same. Upright shoots are thought to have approximately the same concentration on all sides. However, the upright stems frequently bend if shoots are treated with the higher concentrations (Fig. 1 B) or the substances are applied to the soil. In the latter case bending occurs toward light resembling phototropism (20, 38). It is assumed that bending is due to unequal growth rates which in turn are due to unequal distribution of the substance applied regardless of the cause. This assumption is supported by figures in the tables showing amount of growth on convex versus concave sides of stems.

Many varied responses occurred after horizontal tomato plants were treated around the stems with lanolin preparations of the substances. That is, the direction and degree of bending varied with the distance of the treated region from the tip. The influence of the substances applied in this way did not extend throughout the plant as readily as did water solutions applied to the soil or to the base of excised shoots. This fact suggests that different tissues are involved in transporting the substance when it is applied in different ways. When the water solution is added to the soil or applied to the base of an excised shoot, it may readily enter the transpiration stream. If applied to the epidermis the substance must first be handled by the cortex and perhaps it is conducted in both directions by cortical tissues. This was suggested also by earlier work (20, 38) which showed that relative humidity affected the rate of movement up the stem when the substance was applied to the soil but not when lanolin preparations were applied to the epidermis.

Figure 3 shows the relative effectiveness of the three substances and the responses induced by treating at various places along the stem. With this method naphthaleneacetic acid appears to be the most effective and indolebutyric acid the least effective. The responses indicate unequal distribution of the substances as shown when water solutions were used.

Figure 2 shows the effect of applying water solutions of substances to the top of the plant after the tip was removed. The chemicals apparently

moved downward and were unequally distributed as with the other methods of application. To induce positive geotropism by this method much higher concentrations of the substances were required than when the solution was applied to the base of an excised shoot. Also, the time required to induce the response with excised shoots was less than that required where the top of the stump was treated.

Pictures appearing in Figure 2, C and D, show a striking difference between the treated and control plants. Axillary shoots are growing on the controls, but not on the treated plants (19). The position of the roots on the treated plants clearly indicates unequal distribution of the substance in favor of the lower side of the horizontal regions. Note also the swollen appearance around the young part of the treated stems. At this place the concentration was probably too great for best rooting. The two rooted plants shown in the picture at first made a positive geotropic response but the young portion slowly moved upward before the roots appeared. The vertical part finally developed roots around the stem in contrast to only the lower side of the horizontal portion. This response was similar to that described for intact plants treated on the soil and then placed in various positions (Fig. 4).

The failure of leaves to show epinasty when the shoot is inverted, though an effective concentration of the substance is applied, still remains a mystery. It is not greatly unlike the failure of ethylene gas to induce epinasty of leaves on inverted shoots (14). Not only do the leaves of inverted and treated shoots fail to make an epinastic response, but they do the opposite, show hyponasty. Figure 1 shows three tomato shoots pointed in different directions but fed from one reservoir containing growth substance. The upright shoot shows pronounced epinasty of leaves. The inverted stem in high concentration remains straight (in contrast with controls) while the leaf blades and petioles show hyponasty. The stem which was at first horizontal made a positive geotropic response followed by hyponasty of the leaves.

Leaves of plants kept in the dark a few days before treatment were sensitive to much lower concentrations of growth substance than normal plants and also the leaflets and petioles showed more pronounced hyponasty. Leaflets frequently curled so that the edges touched.

Epinasty of leaves on upright shoots was induced by use of a wide range of concentrations of growth substances. The other response, hyponasty, was produced only when the higher concentrations were used.

From the responses described, it can be seen that either the growth substance does not accumulate on the lower side of the leaves as in the case of stems or the tissues of the two organs do not have the same capacity to respond. The upper side of the petiole is capable of growth as is well evidenced by the fact that when a tomato plant is subjected to an atmosphere containing ethylene or other unsaturated hydrocarbon gases

the leaves grow downward exerting a force equivalent to 20 grams or more (14). Therefore, it is difficult to understand why leaves of upright shoots move downward when the substance is applied to the basal end of the stem. The response is the reverse of that produced by horizontal stems. Also if the substance settles to the lower side of the organs why does not the petiole of treated, inverted shoots move upward? The explanations of ethylene-induced epinasty by van der Laan (23) do not help to clarify these responses, because as was pointed out previously his assumption of decreased auxin production does not explain the resulting increased growth.

Another response which seems important enough to mention again is epinasty of leaves and growth of stems induced without the application of any active chemicals. All that is necessary is to induce a geotropic response by placing the plant in a horizontal position, after which it is returned to the original position. The stem soon starts bending back toward its original, vertical position and all of the leaves except two or three of the youngest, promptly show epinasty (Fig. 5, Tables V and VI). The response seems to be associated with increased metabolic activity which in turn is associated with growth curvatures. Denny (16) found an increased production of emanations (probably ethylene) during geotropic response of tomato stems. Possibly there is increased production of natural hormones with increased metabolic activity (41); several other results point in that direction. For example, within 24 hours after the plants are returned to the upright position the nodes associated with epinastic leaves become enlarged and change to a lighter color. If the stem had originally a purplish tinge, it promptly loses this color and turns whitish. Finally white swollen patches develop along the internodes, and then root primordia soon make their appearance in the affected areas.

All of these responses can be induced by the application of synthetic substances to the soil in which plants are growing. Therefore, it does not seem unreasonable to suggest that the unusual responses described for geotropically stimulated plants might have been due to an increased production of natural growth substances.

Du Buy and Nuernbergk (8, 9) measured growth rate on two sides of phototropically stimulated coleoptiles. They concluded that either the illuminated side did not grow or grew less than the shaded side but that the total growth was the same as for non-stimulated coleoptiles.

Dolk (18) studied the influence of gravity on total growth, production of growth substance, and distribution of growth substance in coleoptiles of *Avena* and *Zea*. He concluded that under the influence of lateral action of gravity the total amount of growth substance produced in the coleoptile does not change, but that more goes to the lower side and less to the upper side of the coleoptile. Rotation on the horizontal axis of the klinostat did not affect the growth of the coleoptile.

Schmitz (29) showed that old grass nodes in which no growth substance could be detected finally developed a measurable amount of growth substance when the coleoptiles were geotropically stimulated.

As stated earlier in the paper most of the experiments were performed with tomato as the main test object. This fact, however, should not be taken to mean that other species did not respond to the treatment. All species tested responded very much alike, as shown by Figure 6. The same figure also illustrates the comparative effectiveness of the three different growth substances when used in the same concentrations. It can be seen that the responses induced by the three substances are similar in the lower and upper limits. The main differences appear with medium concentrations where naphthaleneacetic acid was always the most effective. That is, positive geotropism can be induced with lower concentrations of naphthaleneacetic acid than with the indole compounds. Also, more epinasty of leaves appeared with low concentrations of naphthaleneacetic acid than with the other compounds (Fig. 6 C).

Due to natural biological variation between individual specimens, it might be expected that some variation in response would appear when several individuals are treated in a lot with the same concentration of a substance. However, when the plants were grown under the same conditions it was possible to obtain comparable results for several successive days with plants from the same original lots. Figure 7 shows results obtained on two successive days with the same concentrations of naphthaleneacetic acid. The pictures were taken at the start, after two and one-half hours, and after five hours while motion pictures were being made. The responses in the two sets are very much alike.

The motion picture shows that movement of leaves and stems appeared first in the treated shoots and finally in the controls; that is, application of the substances hastens geotropic responses. This holds for the high concentrations causing positive geotropism, as well as for the lower concentrations causing negative geotropism as shown in Figure 7. This same figure shows also epinasty of leaves in low concentrations and hyponasty with high concentrations of the substance.

SUMMARY

The results of experiments involving treatment of intact plants and excised shoots with growth substances show that induced tropisms are conditioned by the relative positions of the organs to gravity. For example, horizontal stems which normally bend away from the earth were induced to curve toward the earth by applying certain concentrations of water solutions of growth substances to the basal end or tip of the shoots. With the same concentrations leaves of upright shoots showed epinasty whereas leaves of inverted shoots showed hyponasty.

Three of the most effective growth substances (naphthaleneacetic acid, indoleacetic acid, and indolebutyric acid) were applied in various ways to intact plants, excised shoots, and plants with tops removed. The plants were placed in various positions (upright, horizontal, and inverted) and the substances were applied to distal parts so that the final effects were systemic rather than local. Regardless of whether the substances were applied to the roots (in soil) of intact plants, to the tops of plants with excised tips, or to the basal end of excised shoots, the final results were similar.

There was some evidence that synthetic growth substances, like natural hormones, were unequally distributed through tissues of shoots placed in a horizontal position. Low concentrations accelerated negative geotropic responses, while high concentrations induced positive geotropism.

Plants kept in the dark for several days lost their capacity to right themselves when placed in a horizontal position. They regained the power to respond to gravity when treated at distal points in various ways with solutions of growth substances.

If the substance was applied to the soil in which plants were growing or to the basal end of excised shoots, it moved upward inducing responses at the tip. If the tip was removed and the upper end of the stump treated with the substance, it moved downward, inducing characteristic responses along the way. Lanolin preparations of the substances applied to the epidermis near the middle of an intact plant induced responses in both directions from the treated region. There was some indication that when the substance is applied to the epidermis its translocation is in the cortical tissue rather than in the transpiration streams.

Measurements of growth rates on all sides of treated shoots indicated that they grew more than controls regardless of the relative position of the shoot with reference to gravity. Growth occurred on the upper and lower sides of geotropically stimulated stems (horizontal) but the convex side elongated more than the concave side. Horizontal shoots treated with medium concentrations continued to grow in a horizontal position. Measurements in these cases indicated approximately equal growth rates on upper and lower sides of the stem, but total growth (sum of the two sides) was greater than that of controls exhibiting negative geotropism.

Measurements of non-treated horizontal stems, turned 180° at intervals, indicated that growth occurred on both sides of the stem but in all cases the lower side grew faster than the upper side. Total growth, however, was greater than that of controls which were not turned after being placed in a horizontal position.

Motion pictures were used to record details of responses while the plants were in treatment.

Epinasty of leaves was induced without chemical treatment by placing

the plants in a horizontal position for 24 hours and then returning them to their original position. Epinasty occurred in all but two or three young leaves as the stems grew back toward the vertical position, indicating redistribution or increased production of natural growth substance or both. The plants finally appeared like those growing in soil to which synthetic substances have been applied.

Five species (tomato, sunflower, cosmos, marigold, and tobacco) were used in the course of the experiments. The results were similar for all species.

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OBSERVATIONS ON THE GERMINATION
OF LETTUCE SEED

A. E. GRIFFITHS

This paper presents a few of the important chemical changes taking place in lettuce seed during germination. No attempt has been made to cover the entire field of metabolic activity, but rather to emphasize those factors related to the hydrolysis of fat. In the case of the lettuce seed, as with many other kinds, fats and oils make up the greater part of the food reserve. Proteins are also important as reserve materials, but there is no starch.

The fats are stored as globules, almost entirely in the two cotyledons of the embryo. However, there may be a little present in the hypocotyl, and in the region of the undeveloped plumule. At the onset of germination the globules first begin to break up in this region, becoming smaller and smaller and finally disappearing completely. Until the second day of germination, minute globules of fat may be found in the newly developed portions of the elongating radicle and hypocotyl. Whether they are transported there as such or are resynthesized is not known. These globules were never observed after the second day of germination.

PREVIOUS WORK

Sachs (8) was one of the first workers to give us qualitative microchemical methods. These methods have since been developed and elaborated by Tunmann (10) and others. By their use we are enabled to establish the presence or absence of minute quantities of organic and inorganic materials in plant tissue. Also, under some conditions, the relative amounts of these materials may be approximately determined. Qualitative microchemical methods are particularly useful in the study of food reserves in single seeds. Faust (5), working with seeds of *Populus grandidentata* and *P. tremuloides*, demonstrated that results obtained from individual seeds were comparable with the results obtained from other seeds of the same sample. Choate (1), Toole (9), and Reuhl (7), working with many different kinds of seeds, have demonstrated that evidence obtained by qualitative microchemical methods very closely agrees with that obtained in the usual macrochemical way. They suggest that crucial points should always be checked quantitatively.

During the past century much excellent work has been performed in establishing the physiological and chemical processes involved during the germination of seeds. Sachs (8), using microchemical technique as early

as 1859, noted that starch appeared in the hypocotyl of oily seeds soon after the germination processes were under way. Also, sugars appeared in various organs of the embryo during the early stages of growth. Detmer (3), in a comprehensive review of the work prior to 1880, organized the known facts in regard to the breakdown and disappearance of such reserve substances as fats, and the appearance of starch, sugar, and other respiratory substrates in various parts of the embryo. Mesnard (6) noted that, as the radicle of some oily seeds commenced to elongate, the oil was carried into the hypocotyl axis. The globules rapidly became smaller and finally disappeared altogether. He did not correlate the appearance of sugars with this, but did notice that the acidity of the embryo increased during the early part of germination. Later, Toole (9), working with maize, found that the globules of fat become much smaller and more dispersed as germination progresses, and, paralleling this change is an increase in the acidity of the embryo. He also observed a rapid increase in reducing sugar.

Eckerson (4), in work concerned mainly with after-ripening processes, has definitely shown that there is a relationship between fat hydrolysis and the appearance of sugars. Her work also indicates that acidity changes within the embryo are closely related to metabolism during germination, probably through effects on the enzyme activity of the seed and on the water absorbing capacity.

Reuhl (7) presents a review of the findings in the field of seed physiology which includes much of the work performed prior to 1936. Her conclusions in regard to the rôle that fat plays in the metabolism of germinating seeds are drawn from microchemical and macrochemical observations with germinating mustard seed. In no case was she able to find that the oil reserves had been converted into sugars at the beginning of germination. In contradiction to most previous work, she points out the probability that fatty acids may serve directly as material for respiration.

MATERIALS AND METHODS

The seeds of *Lactuca sativa* L. used in this study were of two types: a white-seeded variety, known as White Boston, Cornell #43, and the black-seeded variety, Grand Rapids. Both were produced during the summer of 1936. Vitality in both cases was exceptionally high, percentage germination being 96 and 98, respectively. The seeds were carefully stored in sealed containers at a temperature of 10° C. until used.

All seeds were germinated in the dark, on cotton, in petri dishes at a temperature of 20° C. Samples for the various tests were removed at four- or eight-hour intervals as the case might be.

When making moisture determinations, the seeds were removed from the germinators, carefully dried between paper toweling to remove excess moisture, weighed and placed in an 80° C. vacuum oven for 24 hours. At

the end of that time they were weighed again and the percentage moisture determined on a dry weight basis.

MICROCHEMICAL TESTS

Acidity. The indicators proposed by Clark (2, p. 41) were used in making all pH determinations. These indicators were chosen because they are relatively free from protein and salt errors. The colors exhibited by the tissue when mounted in drops of the indicator were compared with phthalate-NaOH and KH_2PO_4 standards. Crucial points were determined also by the glass electrode method. The colorimetric determinations check to 0.2 of a pH unit.

Fat. 1, Sudan III, red color; 2, Myelin formation; 3, Saponification.

Sugar. 1, Flückiger's reaction, red precipitate of cuprous oxide; 2, Phenylhydrazine reaction.

Lipase activity was determined according to standard laboratory methods, except that toluene was used rather than formalin as an antiseptic in the substrate.

ACIDITY OF LETTUCE SEED DURING GERMINATION

Several investigators working with seeds other than lettuce have found that concomitant with the increased activity in germinating seeds there is an increase in acidity. Whether this increase in acidity is a result of increased lipase activity or whether the lipase activity is the result of increased acidity is not known. However, it is obvious that the two phenomena are intimately related, and a study of the acidity changes during early germination may throw some light on the subject.

Normal lettuce seeds were placed to germinate under the conditions described above. At intervals of four hours three seeds of each of the two varieties were removed; free-hand sections made, and the pH of the three important parts of the embryo determined. These determinations were continued until the 60th hour, at which time the seedlings were 4 to 5 cm. in length.

The dry seeds of both varieties have an average pH value of from 5.8 to 6.0 (Fig. 1). The cotyledons, hypocotyl, and radicle are comparatively the same. As germination progresses the principal increase in acidity takes place in the radicle. Possibly this is due to an influx of fatty acids from the cotyledons during the early phases of germination. The increase in acidity continues until the 48th hour at which time the pH is 4.6 to 4.8. Soon after this period the acidity diminishes.

The changes in the cotyledons follow the same general order as those in the radicle but are not nearly as marked. The pH never becomes lower than 5.0.

The hypocotyl changes but slightly in acidity although there are some indications of a low point at the 48th hour.
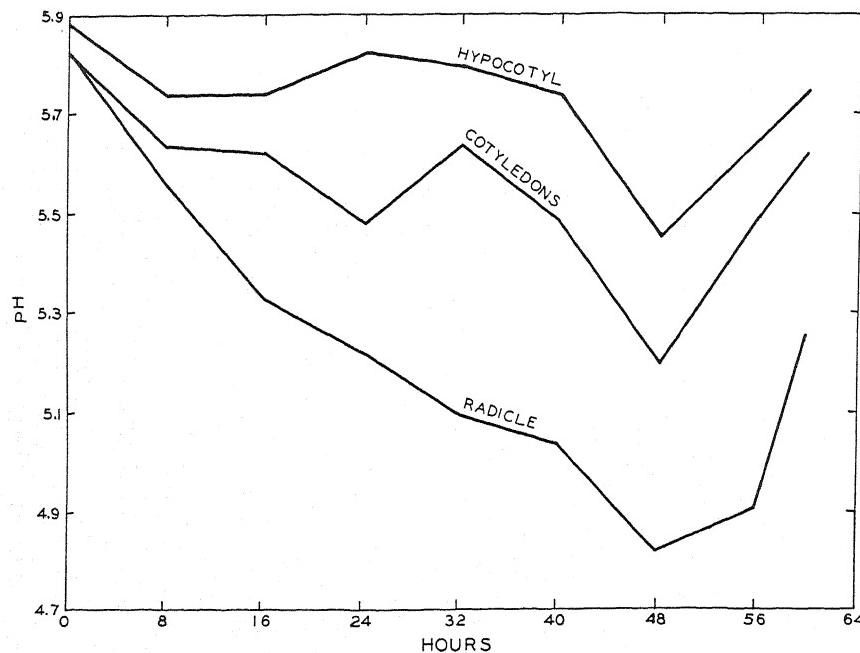
FIGURE 1. Average pH values for White Boston and Grand Rapids
lettuce seed during germination.
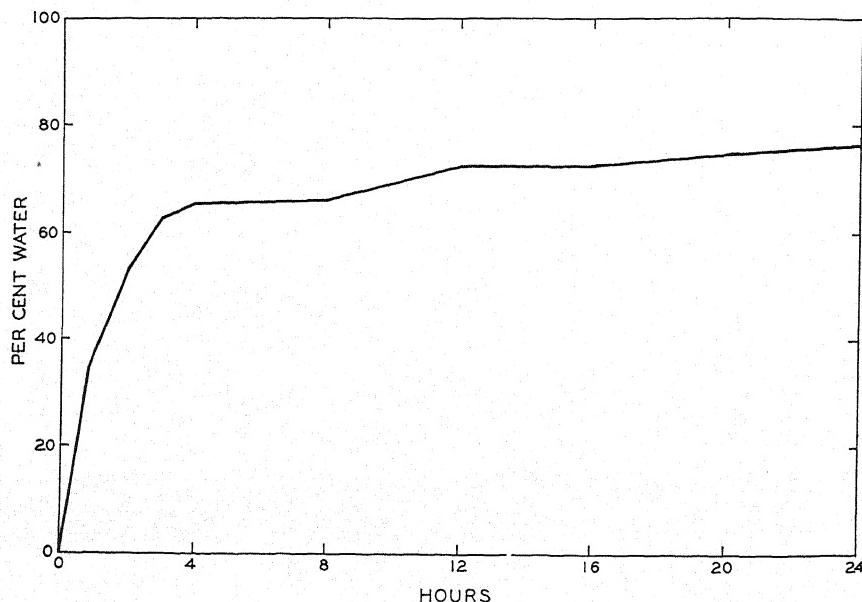
FIGURE 2. Rate of water uptake in lettuce seed during the early stages of germination.
Averages for White Boston and Grand Rapids varieties.

WATER ABSORPTION

It might be expected that as the acidity of the seed increases during germination, the rate of water absorption would also increase. On the other hand, before germination can commence most seeds must have absorbed at least 75 per cent of their dry weight in water. It is interesting to note (Fig. 2) that in the lettuce seed 65 per cent of the water is absorbed in the first four hours. During this same period of time the pH has decreased approximately only 0.1. Even after 12 hours the pH has decreased only 0.2, while the water uptake has approximated 75 per cent of the dry weight of the seed.

The first indication of exterior activity during germination is noticed between the 8th and 12th hours, at which time the tip of the radicle begins to break through the seed coat.

It would appear from these observations that water uptake is not greatly dependent upon the increased acidity of the seed, particularly during the early stages of germination.

FAT METABOLISM

Various investigators have shown that the rate of fat transformation increases with an increase in embryonic acidity; the theory is that lipase is most active as the pH approaches 5.0. Thus the hydrolysis of fats into fatty acids and glycerol is increased in rate as the embryo becomes more acid.

Seeds of both varieties were placed to germinate as before. At four-hour intervals samples were tested for fats. Qualitative microchemical methods were used. For ease in interpretation and presentation an index ranging from 100 (maximum fat) to 0 (minimum fat) was assigned to definite gradations in the observed reactions.

Very little activity was observed during the first 32 hours of germination (Fig. 3). All samples gave strong fat tests, slight differences, if present, not being detected. From the 32nd through the 76th hour the decline in fat content of the embryo was rapid and constant. The 48th hour would represent the approximate median point of highest activity. By the 48th hour, fully two-thirds of the original stored fatty materials had disappeared. It is interesting to observe that this period is also the turning point in seed acidity. After the 48th hour the acidity decreases. It is important to note that the period of greatest activity in fat transformation corresponds to the period of greatest increase in acidity. This might be expected because of the increased amount of fatty acids resulting from the hydrolysis of the fat reserves.

LIPASE ACTIVITY

There are no microchemical tests for lipase. Therefore, it was necessary to resort to standard laboratory methods of determination. The seeds were placed to germinate for definite periods of time. They were then removed,
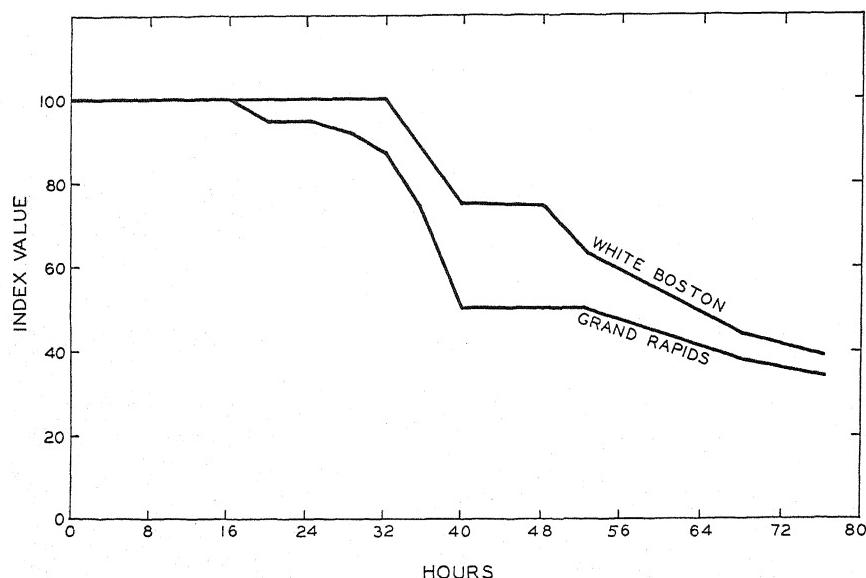
FIGURE 3. Rate of fat loss in lettuce seed during germination.
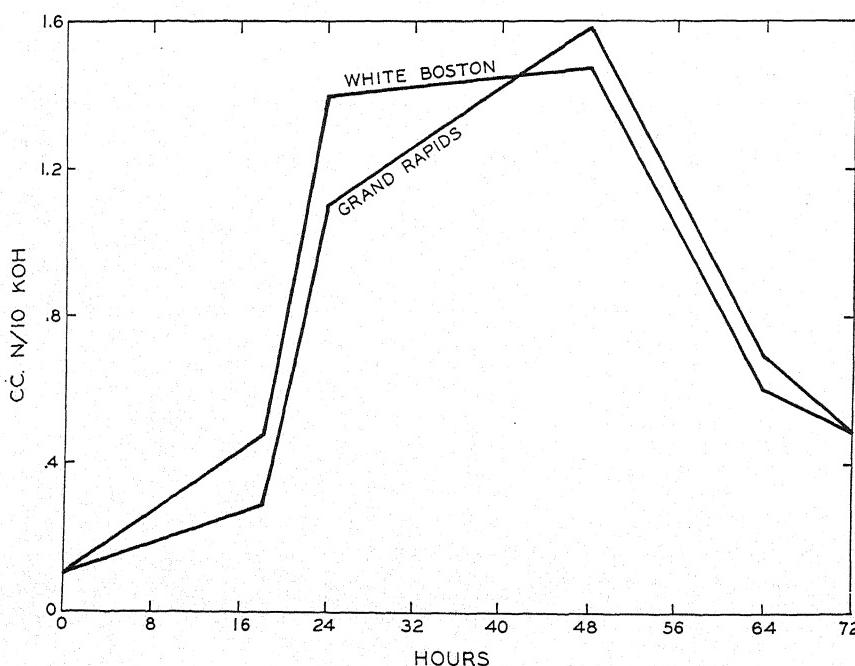
FIGURE 4. Lipase activity in lettuce seed during germination.

dried, ground, and the fats extracted with ether. The residue containing the lipase was then added to definite quantities of a cottonseed oil emulsion. Aliquots of 25 cc. were titrated with N/10 alcoholic KOH. Phenolphthalein was used as an indicator. The emulsion was then incubated for 24 hours at 38° C. and another 25 cc. aliquot titrated as before. The increase in acidity, as indicated by the increased amount of N/10 KOH necessary to bring about the end-point, was taken as due to lipase activity. The data presented in Figure 4 indicate definite increases in lipase activity during the early phases of germination.
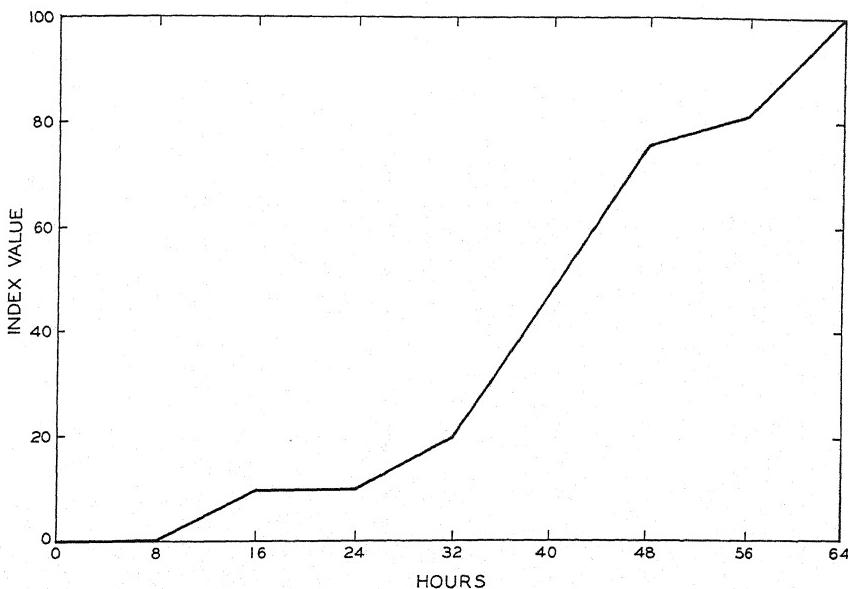
FIGURE 5. Sugar content of White Boston lettuce seed during germination.

Figure 4 shows that there is little or no increase in lipase activity during the first 16 hours, but that there is a sudden increase between the 18th and 24th hours. It will be noted that this sudden change in lipase activity occurs with but a relatively small increase in acidity. One of two conclusions might be drawn from this. Either the acidity change necessary to set lipase into action is very small, or else lipase activity in lettuce seed is not wholly conditioned by the degree of embryo acidity.

Between the 24th and 48th hours of germination lipase activity remains fairly constant at a high level. It is during this period that the greatest transformation of fats takes place. After the 48th hour acidity decreases; the lipase activity drops off and fat hydrolysis begins to slow up, as the end of the reserve supply is approached.

APPEARANCE OF SUGAR

Qualitative sugar determinations were made with free-hand sections of seeds taken at eight-hour intervals. An index of 100 was adopted to represent maximum sugar, and zero to represent minimum sugar. No sugars, either reducing or non-reducing, could be observed in the dry seeds. Very slight increases could be noted until the 32nd hour of germination. From that time on the sugar content of the germinating embryo increased rapidly in indirect proportion to the decrease in fats. Figure 5 shows that here again the period of greatest activity corresponds very closely with the periods of greatest activity for fat hydrolysis, lipase activity, and acidity change.

Practically all of the sugar synthesized from the break-down products of the fatty reserves is in the form of glucose. A small amount of fructose is present, but at no time could sucrose be observed.

DISCUSSION

The foregoing work represents an attempt to clarify and correlate some of the important changes taking place during the germination of lettuce seeds. Conditions such as dormancy or delayed germination may easily have their genesis in the disarrangement of such a balanced system as would seem to surround the transformation of the fatty reserves of lettuce seeds.

This work has been mainly qualitative in nature. For definite proof of the general relations found therein, it should be followed by quantitative study. However, these qualitative tests have allowed the development of a rather clear and nearly complete picture in a shorter period of time than would be possible under quantitative conditions.

SUMMARY

1. It has been possible to develop a relatively clear picture of the conditions surrounding fat metabolism in lettuce seed by microchemical means.

2. The acidity of the lettuce embryo as a whole increases from an approximate pH of 5.8 to 4.9 during the first 48 hours of germination. After that the acidity decreases.

3. The hypocotyl is always more basic and exhibits less change than either the cotyledons or the radicle. The radicle is the most acid part of the embryo.

4. Water absorption in the lettuce seed is rapid, enough being absorbed in the first four to eight hours to start germination. Rate of water absorption does not appear to be dependent upon acidity changes in the lettuce seed.

5. The most rapid hydrolysis of fat occurs between the 32nd and 64th hours.

6. The most rapid increase in sugar occurs between the 32nd and 64th hours.

7. Lipase is most active in the lettuce seed between the 24th and 48th hours.

8. Lipase activity increases prior to the increase in acidity. It would seem that the lipase is relatively independent of the acidity in these early stages of germination. However, lipase activity is maintained at its highest level only as long as the acidity remains high. After the 48th hour, as the acidity decreases and the fat content becomes definitely lowered, the lipase activity falls off. Apparently lipase can begin its work irrespective of the pH of the embryo, but as fatty acids result from the hydrolysis of the reserve material, the enzyme becomes increasingly active.

9. The most active period during the germination of the lettuce seed is centered around the 48th hour. From then until the 72nd hour the curve of activity, as regards the utilization of reserve materials, is a declining one. By the end of the third day the seedling is from 4 to 5 cm. in length, and, given the proper conditions, is capable of manufacturing its own food supplies.

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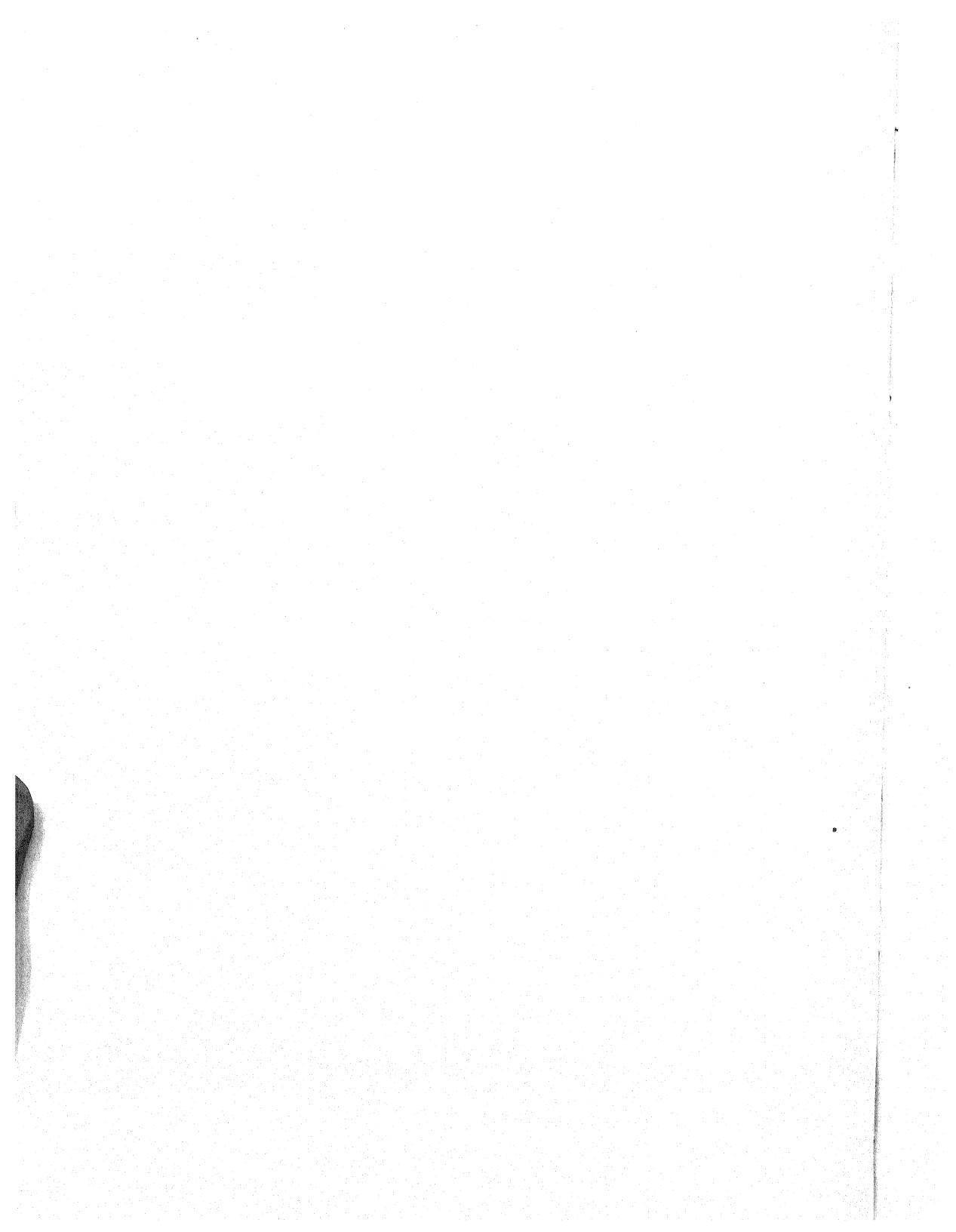
A RAPID METHOD FOR DETERMINING THE VIABILITY OF DORMANT SEEDS

Florence Flemion

Germination has been the usual method of testing viability. From three to twenty-one days are required for the complete germination of cereals and garden seeds. There is, however, no official seed testing method for the dormant forest and fruit-tree seeds. Before these seeds can be tested intact they must be subjected to long periods of after-ripening (from two to six months) in a moist medium at low temperature, then subsequently transferred to 20° to 22° C. before germination occurs. Considerable time and equipment are necessary to furnish optimum conditions for the germination of every kind of seed. It is therefore very desirable that a rapid method of testing viability of seeds be available in order that determinations can be made quickly prior to being subjected to the required periods at low temperature which are essential for germination.

The direct inspection or cutting method merely gives the percentage of seeds containing embryos. As early as 1876 Dimitriewicz (20) turned to chemicals as a means of detecting viability. By applying sulphuric acid on cut sections of grain seeds, he observed that a deep rose color appeared in viable seeds within two to five minutes, while in poor seeds the color appeared only after 15 minutes. Lesage (48) reported that he could detect viability within four hours by soaking *Lepidium sativum* seeds in certain concentrations of potassium hydroxide.

In 1906 Qvam (57) reported a relationship between germination capacity and the respiratory activity of grains. Darsie *et al.* (12) concluded that viability could be ascertained by the amount of heat given off under conditions suitable for germination. Waller (69) and Fraser (33) found that an electrical response ("blaze current") of seeds could be used as an indication of viability. Fick and Hibbard (26) reported that a correlation existed between electrical conductivity and seed viability. Niethammer (54) attempted to find a correlation between sugar content and viability. Many investigators (53, 38, 54, 15, 47, 2) have determined the activity of various enzymes especially catalase in seeds. There is always considerable catalase activity when there is a full manifestation of vitality in seeds. Difficulties, however, arise because dead seeds often contain catalase (68). Davis (16) found that by soaking seeds in water at a high temperature the activity of catalase decreased in dead seeds but increased in viable seeds. In general, catalase activity has not been found to be a satisfactory index of the germinative capacity of seeds (11, 45, 51).

Neljubow (52) tested many organic dyes on seeds. These dyes do not penetrate living tissue but readily enter dead tissue. Indigo carmine 1:2000 was found to be the best. The relationship between the degree of staining and viability must be worked out for each species (44, 21, 59, 50). Sakata (58) found that viable seeds when soaked too long in indigo carmine are also stained. Gurewitsch (39, 40) and others (23, 70) used para- and ortho-dinitrobenzene on seeds. The method (49) depends upon the ability of living cells through respiratory activity to reduce the chemicals into compounds which give a characteristic color reaction in the presence of ammonia.

Scheurlen [as quoted by Eidmann (25)] found that anthrax bacteria were able to reduce tellurium and selenium salts to form free tellurium and selenium respectively which could readily be identified by their color; Klett (25) found that the rate of reduction increased with increased vitality of the bacteria. Sakata (58) and Hasegawa (42, 43) reported satisfactory results with these compounds on seeds of Japanese pine, cedar, and cypress. Eidmann (25) preferred the selenium salts.

Doroshenko (22) utilized the plasmolytic method for determining the germinating capacity of seeds.

Many seed investigators have in the course of their experiments worked with excised embryos. They report that dormant embryos when placed on moist filter paper at room temperature fail to germinate normally. This type of slow development has also been observed in cases where dormancy has been induced in embryos not normally dormant (18, 63). In some cases germination actually occurs but the seedlings are sluggish in their growth (11, 10, 28, 29, 63, 46, 62). Often there is merely an enlargement and greening of the cotyledons (7, 19, 41, 17, 27). Recently, Afanasiev (1) suggests that viability of *Magnolia acuminata* seeds can be detected by the presence of a green pigment formed when the endosperm is injured and kept under germinative conditions.

Tukey and Barrett (66) reported that by placing naked embryos in an agar medium under sterile conditions an approximate germination test for peach seeds could be obtained within seven days. This is the procedure used (64, 13, 14, 65) to obtain seedlings from immature cherry and peach embryos. A simpler method for rapidly determining the germinative power of peach seeds has been published (30). By placing excised embryos mixed in moist peat moss at 25° C. the non-viable seeds deteriorated while the viable seeds showed hypocotyl development within five to ten days. The percentage germination thus obtained agreed with the percentage germination obtained several months later when the seeds had been after-ripened at low temperature.

Previously, in the experiments with excised embryos of various rosaceous seeds it was observed that in the case of freshly-harvested peach seeds

80 to 90 per cent germinated (29) while with *Sorbus aucuparia* no germination occurred, but the cotyledons adjacent to the moisture enlarged and developed a green color (27). In extending these studies, it was found that the viability of various dormant seeds (32) could be rapidly tested by observing the behavior of excised embryos when placed on moist filter paper in petri dishes at room temperature. The results thus obtained were comparable to the percentage which germinated considerably later after the intact seeds had been subjected to the required low temperature period.

By the method described in this article viability of seeds can be determined within three to ten days in very dormant seeds and within a very short time in the case of quick-germinating seeds. The percentage viable as well as some indication of vigor is obtainable. While the time required to test the viability of seeds as compared with the germination of intact seeds is greatly reduced, this rapid method requires considerable care and patience for the excision of embryos. However, much time, labor, and expense can be eliminated, especially when the seeds are found by this quick method to be very poor or dead.

MATERIALS AND METHOD

After cleaning, the seeds were stored dry at room temperature. Most of the seeds were collected in the vicinity of the Institute, a few were obtained from outside sources.

The viability of the seeds was tested in two ways. Excised embryos were kept on moist filter paper at room temperature (21° to 23° C.). The behavior of these embryos was compared with the percentage germination obtained by following the procedures recommended in published articles.

The technique for excising embryos varied somewhat. After apple, pear, and *Sorbus aucuparia* seeds had soaked overnight in tap water, a cut was made through the outer and inner coats with a sharp scalpel and the coats removed. The hard outer coats of witch-hazel, *Rhodotypos*, plum, peach, hawthorn, and fringe-tree seeds were removed with the aid of a device used for cracking seed coats (30). The inner coats of these seeds were then removed as in the case of apple after soaking overnight in water. Embryos of Douglas fir and pines are soft and easily injured. After soaking in water, the outer coats of these seeds were removed, several slits made in the endosperm and then upon subsequent soaking in water the embryos usually floated away from the endosperm without any additional assistance. In the case of fringe-tree seeds, several slits were made in the endosperm; then, after being in water overnight, the embryo was removed by spreading the endosperm open at one of the slits. In this way the embryos were not injured.

As the embryos were excised they were placed in water and with a brush or scalpel they were transferred to the moist filter paper. Too much

moisture or too high a temperature is not conducive to good development of the embryos. During the summer months when the temperature in the laboratory was high some of the cultures were placed in a 20° C. controlled oven with good results. For ten pieces of 15 cm. filter paper (#1 W. & R.

TABLE I

COMPARISON OF THE TWO METHODS OF TESTING THE VIABILITY OF SEEDS

Family	Species	Crop*	Experiment started, date	Results, percentage viable	
				Excision method	Germination method
Rosaceae	Apple	1936 " " " "	Jan. 1937 " " " " " " Feb. 1937	93 84 78 91 95	94 71 76 87 86
	Pyrus ussuriensis	1936 "	Feb. 1937 Mar. "	96 92	86 86
	Wild plum	1935	Feb. 1937	96	100
	Sorbus aucuparia	1930 1933 1936 1929**	Feb. 1937 " " " " Mar. "	0 7 100 86	1 6 90 62
Hamamelidaceae	Witch-hazel	1934 1936	April 1937 " "	0 100	0 98
Pinaceae	Douglas fir	1937	Oct. 1937 Nov. " " " " "	33 69 87 88	45 84 51 77
	Pinus densiflora	1928 1937 1937	Feb. 1938 " " Jan. "	0 86 100	0 76 86
	Pinus rigida	1928 1937 1928 1937	Nov. 1937 " " Jan. 1938 " "	0 44 0 40	0 48 0 61
	Pinus thunbergii	1929 1937	Jan. 1938 " "	0 52	0 58

* Seeds stored dry at room temperature.
** Seeds stored in a sealed container at 10° C.

Balston Ltd.) 30 cc. of tap water were used for the rosaceous embryos and only 25 cc. of water for the soft embryos of pine and Douglas fir.

Since embryos only are used for the petri dish test proper corrections must be used if some of the original seeds are empty or contain shriveled embryos.

RESULTS

Seeds Requiring After-ripening at Low Temperature

Apple. Within six days after the excised embryos (*Pyrus malus* L., var. unknown) are placed on moist filter paper at room temperature the non-viable embryos begin to deteriorate while the viable ones appear as when freshly-excised or show some development (Fig. 1 C). Among the viable embryos some may actually germinate but often there is merely some activity in one or both cotyledons. As seen in Table I, the percentage viable in the petri dishes (duplicates of 25) compares satisfactorily with the percentage germination obtained several months later when duplicate lots of 100 intact seeds had sufficiently after-ripened at 5° C. (10).

Pear. The correlation between the behavior of pear (*Pyrus ussuriensis* Maxim.) embryos on moist filter paper and the percentage germination of intact seeds is similar to that of apple. Duplicate lots of 50 seeds were used for the germination tests and one lot of 50 for the petri dishes (Table I).

Sorbus aucuparia L. The viability of European mountain ash seeds of various crops was tested by the two methods with good agreement (Table I). In Figure 1 E the behavior on the sixth day of a non-viable embryo of the 1930 crop and a viable embryo of the 1937 crop is illustrated. Viable embryos remain as when freshly-excised or there is an enlargement of one or both cotyledons associated with a deep green color. Errors may arise if readings are made before the fifth day for seeds low in vitality may appear to develop. However, such embryos soon deteriorate and the coloring, if present, is a pale yellow-green in contrast to the deep green coloration found in good seeds. For the germination tests duplicate lots of 200 were placed at 1° C. (27).

Rhodotypos kerrioides Sieb. & Zucc. The development of excised embryos of *Rhodotypos* seeds gives some indication of vigor as well as of viability. The behavior of embryos of five crops after five days on moist filter paper is illustrated in Figure 1 A. The fall of vitality with age is readily seen in this figure for there is a gradual rise in vigor from the old 1932 seeds to the recently harvested 1937 crop. On the tenth day, embryos of the 1935, 1936, and 1937 crops had long hypocotyl growth while the behavior of embryos of the other two crops can be seen in Figure 1 B. Previous germination tests had shown that seeds of the 1932 crop were dead and those of the 1934 crop were very poor. Those embryos of the 1934 crop which show some development have a pale yellow-green color and lack the vigorous growth found in good seeds. Germination occurs in the more viable embryos.

Witch-hazel. Four methods of determining viability were tested on these seeds (*Hamamelis vernalis* Sarg.). In Figure 1 F the embryos of three

EXPLANATION: FIGURE 1

(Reading from left to right in each case)

A and B. *Rhodotypos kerrioides*. A. Freshly excised embryo and 5-day development on November 10, 1937 of 1932, 1934, 1935, 1936, and 1937 crops, respectively. Note deterioration in typical embryo from 1932 crop, limited development in 1933 crop, and increasing vigor in 1935, 1936, and 1937 crops. B. Embryos of 1932 and 1934 crops after ten days. The 1932 embryo is undergoing destruction by organisms and the 1934 embryo is still considerably behind the 5-day development of embryos from later crops as shown in A. The 1934 embryos have a pale yellow-green color, rarely exhibit any hypocotyl development, and deteriorate rapidly. Embryos which show this type of development are low in vigor and a low percentage of seedlings is obtained when the intact seeds are after-ripened at low temperature.

C. Apple embryos. First, a freshly-excised embryo is shown and then after 6 days on moist filter paper two non-viable ones followed by two viable embryos showing typical greening of cotyledons and growth. Photographed November 9, 1937.

D. *Crataegus crus-galli*. Ten-day development on February 1, 1938. The first three illustrations are of embryos of the 1928, 1930, and 1933 crops, respectively. The embryo of the 1928 crop has undergone marked deterioration while the embryos of the 1930 and 1933 crops are also shown to be non-viable. At the same time the behavior of two viable embryos is shown. At the extreme right in D is an embryo of seeds of the 1936 crop which had been in moist peat moss at 5° C. for one year. Obviously the intact seed had not after-ripened during this time but had remained viable. The viable embryo of the 1935 crop (second from right) will with time also show similar enlargement of the cotyledon next to the moist filter paper. It is readily seen that viable hawthorn embryos do not exhibit the extreme development obtained with viable *Rhodotypos* seeds.

E. *Sorbus aucuparia* embryos. On the left is a photograph of a freshly-excised embryo after which follows a dead embryo of the 1930 crop and a viable embryo of the 1937 crop after six days (November 18, 1937) on moist filter paper.

F. Here are shown (November 18, 1937) embryos of witch-hazel of the 1934, 1935, and 1936 crops, respectively after 5 days. The 1934 embryos are dead and are undergoing deterioration, the 1935 embryos which are low in vitality exhibit very little activity—the cotyledons merely spread apart—while the viable 1936 embryos are growing. In G, H, and J are shown in each case two typical embryos of the 1934 and 1936 crops, the older embryo appearing on the left. The embryos in G have been soaked for 20 hours in a saturated solution of para-dinitrobenzene followed by one-half hour in dilute ammonia and those in H have been treated for 20 hours in 1 per cent potassium tellurite. J illustrates the behavior of embryos after 5 days on moist filter paper. The chemical tests give some color even in the 1934 crop and reactions intermediate between those shown also occur so that it is difficult in many cases to decide which embryos are alive by chemical tests. The results on moist filter paper are much easier to interpret.

K. *Prunus americana* embryos after several weeks on moist filter paper. All these embryos are viable and the various types of development are illustrated. The dead embryos deteriorate rapidly while the viable ones develop slowly.

L. Fringe-tree. The embryo on the left illustrates the deterioration of a non-viable embryo after 4 days and the other photographs illustrate a viable embryo after 4, 6, and 20 days, respectively.

M. Douglas fir. A freshly-excised embryo appears on the left, next a viable and a non-viable embryo after 4 days on moist filter paper.

N. *Pinus rigida*. A freshly-excised embryo, and after eight days a non-viable embryo and two viable embryos exhibiting low and high vitality respectively.

(The coloring of the photomicrographs was done by Miss Flora White.)
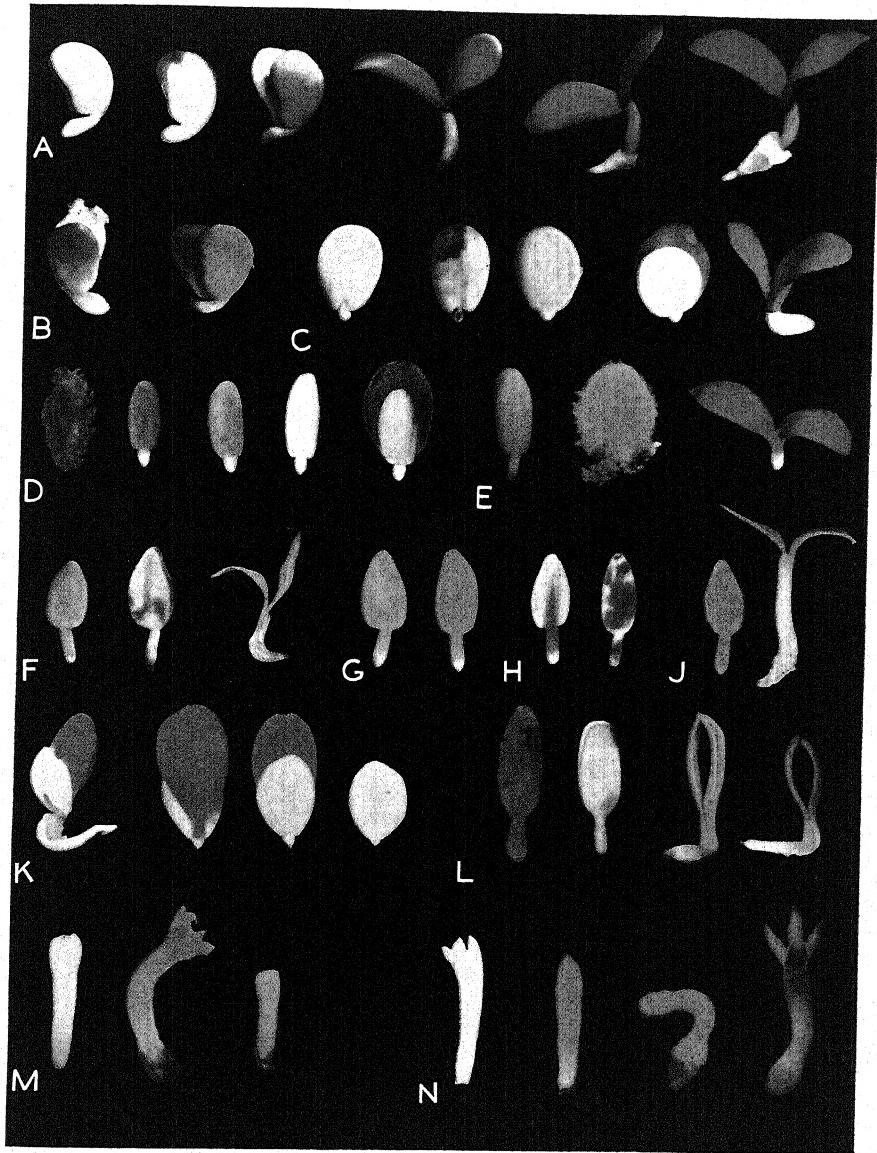
FIGURE 1. Development of excised embryos on moist filter paper in petri dishes at room temperature: K natural size; E, M, and N ×4; all others ×2. (See explanation on opposite page.)
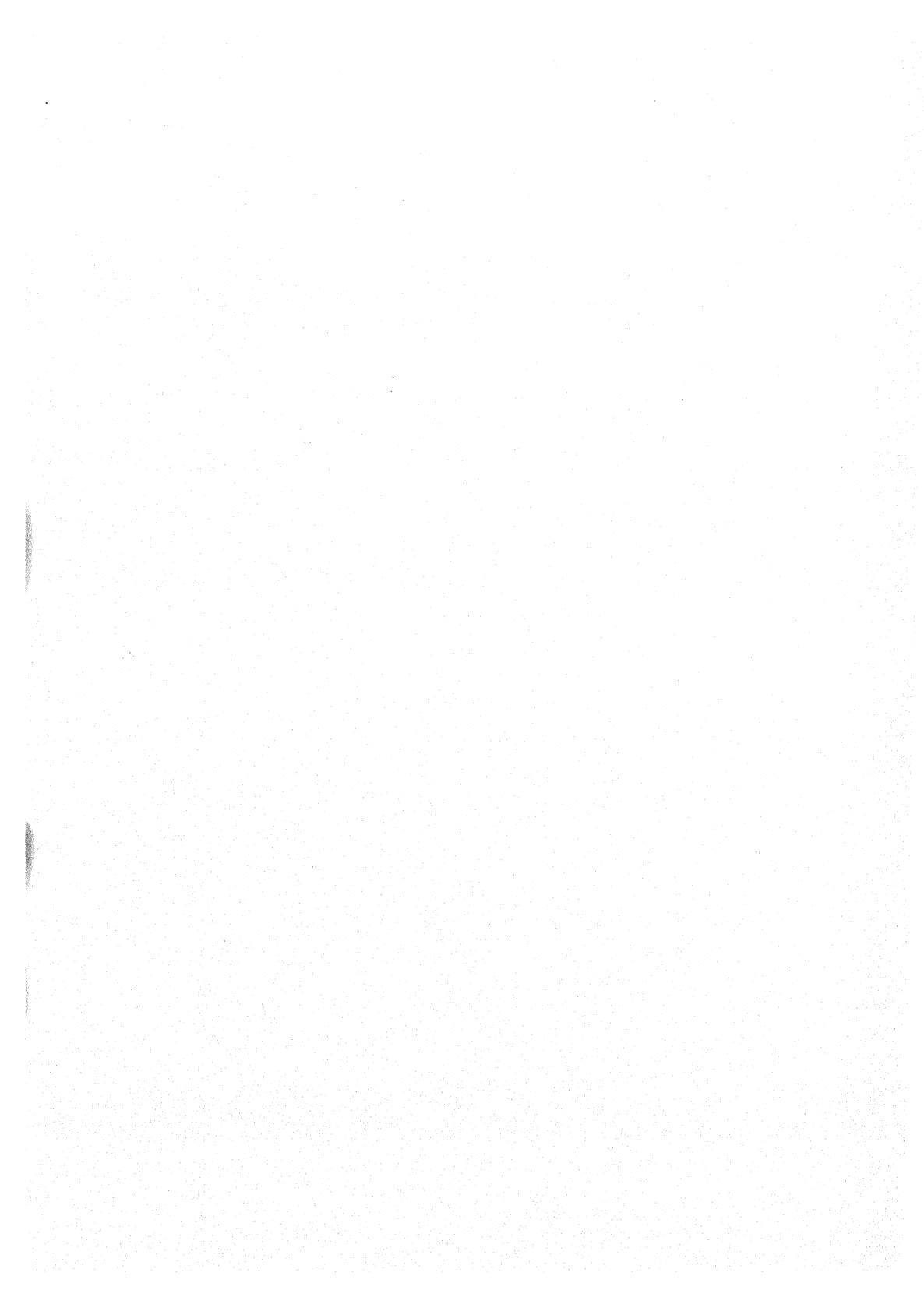
crops after five days on moist filter paper are shown. It is readily seen that seeds of the 1936 crop are viable, those of the 1935 crop are very low in vitality, while the 1934 seeds are dead. The more viable embryos germinate, the dead embryos decay, while those low in vitality exhibit some activity in the cotyledons for water is taken in and the cotyledons spread apart which is a behavior never found in dead seeds. A comparison (Table I) of the behavior of excised embryos agrees closely with the percentage germination obtained after five months at 5° C. The methods suggested by Gurewitsch (39, 40) and Sakata (58) were also used. Excised embryos were used because the seed coats were found to be impermeable to these chemicals. In Figure 1 G, H, and J the non-viable 1934 embryos are on the left and the viable 1936 embryos are on the right. The color of embryos soaked in a saturated solution of para-dinitrobenzene for 20 hours followed by one-half hour in a very dilute solution of ammonia is shown in Figure 1 G. Embryos were also soaked for 20 hours in a 1 per cent potassium tellurite solution. Accurate interpretation was often impossible. Thus with tellurium salts all degrees of coloring between those shown in Figure 1 H were observed. For comparison the differences in behavior of dead and viable embryos after five days on moist filter paper may be seen in Figure 1 J.

Peach. The method of handling peach (*Prunus persica* [L.] Stokes) embryos for rapidly determining the viability of peach seeds has already been published (30). Excised peach embryos are large and require less space if mixed in moist peat moss than when placed on moist filter paper. Either method may be used.

Plum. The various types of development of viable excised embryos of *Prunus americana* Marsh. (wild plum) are shown in Figure 1 K. The percentage germination (37) of one lot of 50 seeds is compared in Table I with the percentage viability of one lot of 25 embryos in the petri dishes.

Seeds with Unusually Thick Woody Coats as Well as Dormant Embryos

Hawthorn. In unpublished work it has been found that the extremely dormant seeds of *Crataegus crus-galli* L. must be pretreated to remove the effect of the seed coat and then subsequently after-ripened at low temperature. Even under optimum conditions five to six months are necessary before germination occurs. After ten days on moist filter paper (Fig. 1 D) the excised embryos of the 1928, 1930, and 1933 crops show signs of deterioration. Previous germination tests have shown that seeds which have been stored dry at room temperature for three or four years are no longer viable. Viable embryos may remain intact as when freshly excised or the lower cotyledon may enlarge and develop a green color. Such behavior of viable embryos is shown in the figure. Incidentally, the illustration shows quite clearly that an intact viable seed receiving no pretreatment for eliminating the effect of the seed coat had not been able to after-ripen even

after one year at low temperature. A partially or fully after-ripened excised embryo shows rapid development within a few days on moist filter paper. More detailed experiments which are now in progress will be published later.

Seeds which Are Not Very Dormant but Respond to Low Temperature

Pines. Within three days after excised embryos of *Pinus densiflora* Sieb. & Zucc., *Pinus rigida* Mill., and *Pinus thunbergii* Parl. are placed on moist filter paper, some indication of their viability is obtainable. At this stage the viable embryos show some development while the dead ones are deteriorating. The results in Table I represent averages of duplicate lots of 25 or 40 seeds in the petri dishes and of duplicate lots of 100 seeds planted after two months at 5° C. (3). The behavior of embryos in a lot of *Pinus rigida* seeds containing viable seeds, dead seeds, and seeds with low vitality is shown in Figure 1 N. Differences in vigor are noted by the extent of the development of the embryos during the petri dish test.

Douglas fir. Results comparing the two methods of testing viability of *Pseudotsuga taxifolia* Brit. are shown in Table I. Due to the limited amount of material, these averages were obtained from a relatively small number of seeds. The illustrations in Figure 1 M are of a freshly-excised embryo and of two embryos—one viable and one non-viable—after four days on moist filter paper.

Seeds with Epicotyl Dormancy

Fringe-tree. There is a type of dormancy (4, 6, 36) whereby the root grows at a warm temperature but the shoot appears only after a period at low temperature. In unpublished work, it has been found that fringe-tree (*Chionanthus virginica* L.) seeds belong to this group. The behavior of the excised embryos is shown in Figure 1 L. The non-viable seeds deteriorate rapidly while the viable embryos develop slowly. The germination results are not yet complete so a direct comparison of the two methods cannot at this moment be shown in actual figures. However, in a previous preliminary test no germination was obtained from lots which had been shown by the petri dish method to be non-viable. These seeds lose their vitality very rapidly when stored dry at room temperature. Storage experiments are now in progress. This rapid method is being compared with germination tests in determining the optimum storage conditions for the retention of vitality.

Quick-germinating Seeds

Hemp. Seeds of two types (*Cannabis sativa* L., var. unknown) viable and artificially killed, were soaked for ten hours in tap water at 30° C. Some were planted, some placed directly on moist filter paper, and some

were excised then placed on moist filter paper. These seeds germinate very quickly but the results from the excised embryos were produced more rapidly, especially in the case of the killed seeds. Upon excision it was noted that the killed embryos were of a different color and had not imbibed much water compared with the viable embryos. After 24 hours the dead embryos appeared as when excised while the viable embryos had germinated. Differences between weak and vigorous seedlings could be detected more quickly in the excised embryos than in the intact seeds.

DISCUSSION

A rapid method of determining the viability of seeds is especially important for use with dormant seeds since in the case of seeds which germinate readily, viability can be determined in a relatively short time. Dormant seeds require various procedures to induce germination (8, 9). Thus some seeds require a more or less prolonged period at low temperature during which changes take place in the seeds (after-ripening) before good germination occurs (24, 55). In some seeds after-ripening at low temperature cannot properly proceed unless the very hard and impermeable seed coats are pretreated either by acid or by long periods in a moist medium at high temperature (61, 5, 56, 34, 35). The percentage germination can be considerably increased in certain seeds (60) when a short period at high temperature precedes the period at low temperature. Other seeds are not so dormant and may even germinate at room temperature (3, 31) but a prompt and often more complete germination is obtained when the seeds are subjected to a short period at low temperature. In still another type of dormant seeds the root can emerge at room temperature but the shoot will not appear unless the seeds receive some low temperature treatment (4, 6, 36). The experiments with excised embryos were designed to include examples of seeds representing these various types of dormancy. The results have shown that the observation of the behavior of excised embryos serves as a good test for viability of seeds. The method has several applications such as testing viability of seeds prior to planting, determining whether the seed is retaining its viability under given storage conditions, or ascertaining whether the viable seeds are after-ripening at certain temperatures.

The results show that no matter how dormant a seed may be the excised embryo usually shows some development when placed on moist filter paper. The embryo is therefore not incapable of growth but for some reason does not develop in the intact seed until proper after-ripening processes have occurred. The various types of seeds studied differ in the extent of development shown by the excised embryos for some grow to a greater extent than others. Excised embryos of peach and *Rhodotypos* actually germinate under these conditions while apple and others may germi-

nate but often there is merely some activity in one or both cotyledons. Dormancy does not appear to be due to the lack of ability of the excised embryo to imbibe water and develop but rather to the degree of development possible or the force and vigor behind such development. Thus growth is very sluggish in these dormant embryos. It has been shown (28, 13, 29, 14, 65, 63, 62) that germinated non-after-ripened embryos when subsequently transferred to soil will develop plants but do not, however, grow at a normal rate; they exhibit dwarfish characteristics for a number of months before apparently normal growth takes place.

Von Veh (67) has suggested the production on a large scale of fruit-tree seedlings from excised embryos. Results in this laboratory as well as those of Davidson (13, 14), Tukey (65), and Koblet (46) indicate that seedlings resulting from non-after-ripened embryos lack vigorous growth for a considerable period of time. Subjecting intact seeds to a period at low temperature brings about conditions which make for vigorous growth. By subjecting the dwarfish plants to low temperature the period of retarded growth can be appreciably shortened. Thus the beneficial effect of low temperature can be utilized by subjecting the seedlings (14, 65) produced from non-after-ripened seeds to low temperature as well as by low temperature treatment of the seeds. Peaches and *Rhodotypos* have been grown in the Institute gardens to the fruit stage and have given trees comparable to those obtained from after-ripened seeds.

SUMMARY

Experiments with a variety of different types of dormant seeds have shown that the viability of such seeds can be determined in from five to ten days by observing the behavior of excised embryos on moist filter paper in petri dishes at room temperature (21° to 23° C.). Non-viable seeds deteriorate under these conditions while viable embryos show various types of development. Results obtained by this method agree well with actual germination tests.

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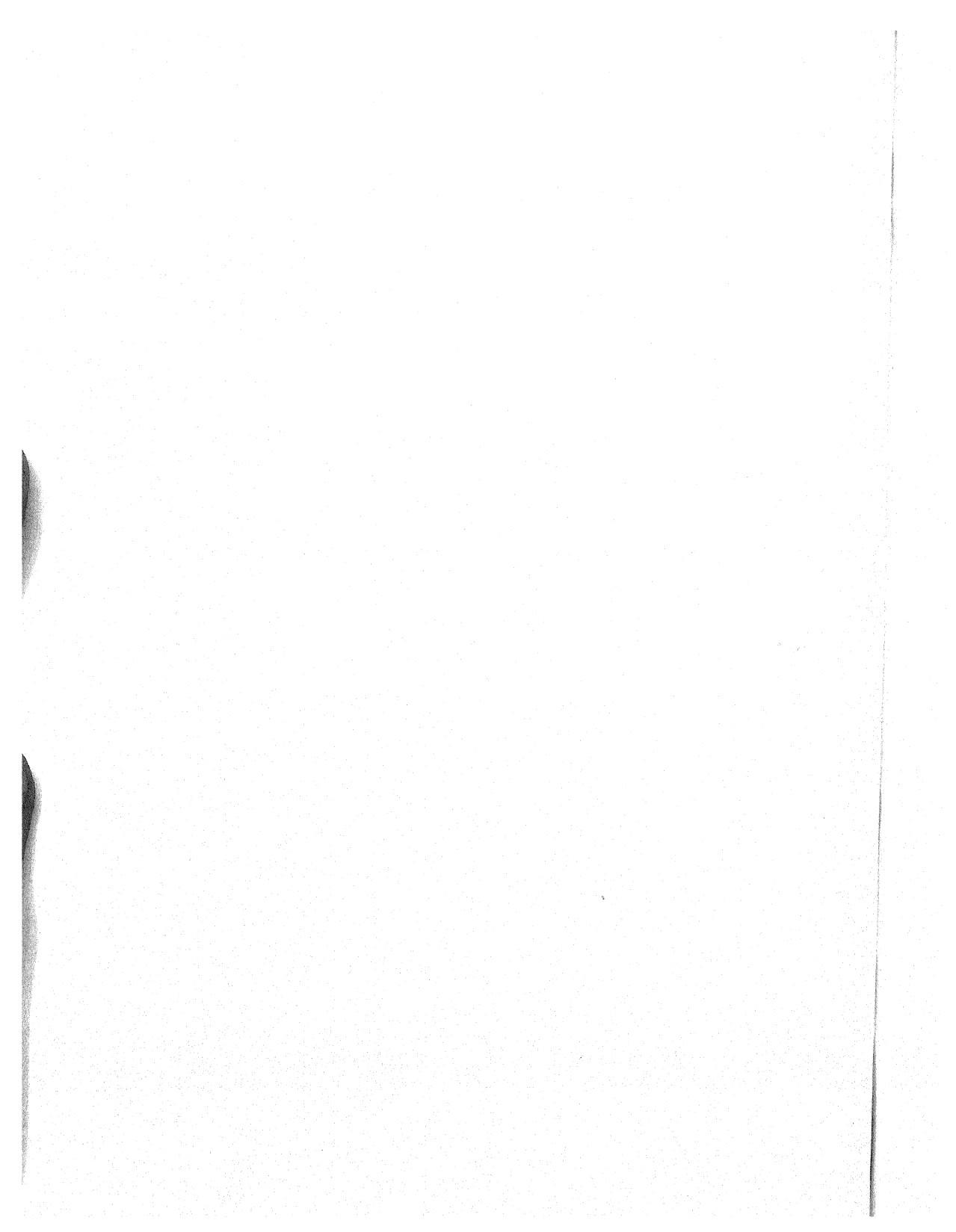
SOIL CHARACTERISTICS IN RELATION TO DISTANCE FROM INDUSTRIAL CENTERS

M. M. McCool and A. Mehlich

Disagreements and controversies relative to the action of sulphur dioxide from industrial centers and metropolitan areas on soils and vegetation were brought to our attention recently. In order to obtain additional information on these questions the Camden, New Jersey, Philadelphia, Pennsylvania, East St. Louis, Illinois, and St. Louis, Missouri, areas were selected for study, areas considered to have been exposed to sulphur dioxide over long periods of time. They also provided soils varying widely in buffer capacity and fertility. There are available much data relative to the sulphur dioxide content of the atmosphere at or in close proximity to the various stations from which samples were taken, as determined by the Air Hygiene Foundation (17) of the Mellon Institute, Pittsburgh, Pennsylvania.

It is assumed that in general the dosage of SO_2 to which the soils of both areas have been exposed during past years decreases with the distances from the centers of the cities. This is probable because the land about both cities is comparatively level and without any points of high elevation. This assumption is confirmed by the SO_2 determinations of the Air Hygiene Foundation. It is also probable that the amount of sulphur brought down to the soil by rain decreases as the distance from the cities increases.

The results of the investigations on the pH values, titratable acidity, base or ionic exchange, exchangeable calcium and magnesium, and the sulphur, sand, silt, and clay contents of soils are presented in this paper. Certain plant relationships are considered in another report.

METHODS

Collection of soil samples. Soil samples were collected in the Camden-Philadelphia area from sites which had remained undisturbed for many years, as revealed by the age of tree and shrub growth. Care was taken to collect samples from those areas which showed similar vegetative growth. The sites available for the most part were small, approximately 40 × 80 feet. Three to six cuttings were taken from each area by means of a small spade. The samples in general were collected according to the following scheme: zero to 1 inch, 1 to 3 inches, 3 inches to the subsoil, subsoil to 12 inches, 12 to 15 inches, and a composite sample of the surface soil. The soil survey report of the Camden area (3) was utilized in the

selection of areas to be sampled. For samples which were collected outside of this region and for which no satisfactory soil map was available, classification was based on comparisons with those described and mapped in adjacent areas. Samples from the East St. Louis, Illinois, and St. Louis, Missouri, areas were collected in a similar manner and were classified on the basis of soil reports (5, 13, 16). Information with reference to flooding was obtained from the sanitary district office of East St. Louis.

Determination of pH. A glass electrode was employed in the measurement of pH values. Soil: water suspensions (ratio 1:2.5) were prepared, stirred, and the determinations made after one-half hour.

Determination of titratable acidity. Sixty grams of soil were shaken in a motor-driven shaker for one-half hour with 150 ml. normal barium acetate. The filtrate was titrated with 0.1 N NaOH, phenolphthalein being employed as the indicator. The results are reported as ml. 0.1 N NaOH required for filtrate from 100 grams of soil.

Determination of base exchange capacity. Five to ten grams of the fine textured soils were shaken for one hour (sands one-half hour) with 250 ml. neutral, normal potassium acetate, digested on the steam bath for one hour, filtered through an 11 cm. Buchner funnel, washed with an additional 250 ml. of potassium acetate and a few ml. of normal potassium chloride. The soil was then leached with alcohol and water until the leach was free of chlorides. The adsorbed potassium was then replaced with 500 ml. of 0.2 N ammonium carbonate. The filtrate was evaporated to dryness on the steam plate, the residue (consisting of potassium carbonate) taken up with about 200 ml. distilled water. The solution was titrated with an excess of 0.1 N HCl, the indicator being bromo-thymol blue. Carbon dioxide was removed from the solution by boiling and the excess acid titrated with 0.1 N NaOH. (Where exchangeable bases were to be determined, the extracting solution was neutral, normal ammonium acetate.) The base exchange capacity was then determined by leaching the ammonium acetate-extracted soil with 500 ml. of 0.5 N KCl and after washing, the potassium replaced with 0.2 N ammonium carbonate. Base exchange capacity is expressed as m.e. per 100 grams of soil.

Determination of sulphur. The sulphur content of the soils was determined by adding to five or ten grams of soil in a crucible, depending upon the amount of organic matter, and intimately mixing therewith, an excess of alcohol-saturated magnesium nitrate. The crucible was tilted so as to allow for an ample supply of air and the alcohol ignited. External heat was gradually supplied by means of a Bunsen burner just after the alcohol had been oxidized and the surface of the soil broken in order to admit oxygen. The flame was gradually raised and the soil ignited to red heat. After cooling, 10 cc. of concentrated HCl were added, the mixture well stirred, and the HCl allowed to evaporate on a hot plate. The soil was then again

taken up with 10 ml. of HCl (1:1), heated, the soil broken up, placed in a filter, washed, first with dilute (1:10) HCl and then with water. The total volume did not exceed 150 ml. The sulphur was then precipitated as barium sulphate and the amount of sulphur determined either by means of the Parr turbidimeter or by weighing.

Determination of soil fractions. The method of Bouyoucos was used for the determination of the sand, silt, and clay fractions (1).

Determination of exchangeable calcium and magnesium. The calcium and magnesium in the ammonium acetate extract was determined by the method of Schollenberger and Dreibelbis (19).

The physical analysis, pH, and titratable acidity determinations were conducted on all samples (3 to 6 borings) for each station. The total number of samples collected amounted to more than 1200. The base exchange capacity of the samples of two borings was ascertained. The exchangeable bases and total sulphur values represent one boring per station. In Table I

TABLE I

COMPARISON OF AMMONIUM ACETATE AND POTASSIUM ACETATE METHODS FOR THE DETERMINATION OF THE BASE EXCHANGE CAPACITY OF SOILS

Soil No.	Ammonium acetate method		Potassium acetate method m.e.	pH of soils	Titratable acidity ml. 0.1 N NaOH per 100 grams soil
	Soil saturated with				
	N CaCl$_2$ m.e.	0.5 N KCl m.e.			
100	1.7	1.9	1.9	4.99	13.5
102	4.4	4.3	5.0	4.76	33.0
105	7.7	7.2	8.0	6.92	5.3
104	8.8	8.5	9.7	5.26	31.2
106	12.2	12.6	—	5.38	41.4
107	13.8	13.7	—	5.82	31.9
101	17.5	16.9	17.4	5.15	96.0
103	20.3	21.2	20.6	5.06	122.0

the results of two methods for the determination of the base exchange capacity in soils are compared. It will be noted that the ammonium carbonate titration method compares favorably with the calcium chloride saturation method.

LITERATURE REVIEW

Wieler (21) drew attention to the earlier view that acids from smelters which fall on the soil are taken up by the roots of plants resulting in injury. He set forth his decalcification theory, namely that acids which come into contact with the soil unite with bases, which in turn are washed out of the ground resulting in a lack of bases, especially calcium. Areas denuded by smelter smoke were used by him as experimental plots. He found the use of lime to be effective in reclaiming the soils for the production of pine, spruce, oak, maple, beech, and beans.

Haselhoff *et al.* (10) critically reviewed much of the early literature on this subject including that of Wieler and Stoklasa. They concluded that repeated fumigations do not significantly increase the sulphuric acid content of soils. On cultivated soils the effect of fumigation on soil acidity is nil, although in forest soils a gradual loss of bases takes place if these soils are exposed to high SO_2 fumigations for a long time and this only on very poor soils. In extreme cases on poor soils the activity of micro-organisms may be affected.

Wieler (22) in replying to Haselhoff *et al.* quoted the results of Niggemeyer in the Ruhr region where the amount of SO_3 falling on a square meter of soil annually ranged from 80 to 90 grams and in other parts of Germany it amounted to 13, 33, 87, and 98.6 grams. He also reported normal growth of barley where lime was used on soil made unproductive by smoke injury.

Wilson (23) reported the quantity of sulphur which reaches the soil in rainwater at Ithaca, New York, to amount to 26.9 pounds per acre annually.

Crocker (6) called attention to reports on the amounts of sulphur deposited by precipitation in industrial centers of England and in different places in the United States and in addition to reports dealing with loss of sulphur from soils through leaching and crop removal.

Eaton and Eaton (8) reported a deposition of 46.04 pounds per acre per year at Liberty, Indiana, and 227.45 pounds at Chicago, Illinois. The largest amount of sulphur was found in the winter months, a decrease in the spring, a minimum amount in the summer, and then an increase in the autumn.

According to Kelley (12) smelter fumes have not affected the pH values of the soil in the Philadelphia, Pennsylvania, area. The results reported by Fraps and Fudge (9, p. 1016) are of interest. They buried 18×24 inch glazed tile in the ground, filled them with Lufkin fine sandy loam, and treated the soil to a depth of five inches with different amounts of sulphuric acid and sulphur. They recorded the changes in pH values over a five-year period and found maximum acidity of the surface soil developed within ten weeks and that of the next two or three inches within four months after treatment. The acidity of all treated soils then slowly decreased. "Penetration of acidity in excess of two or three inches occurred only in series in which the surface soil became so acid that no plant growth could occur."

Byers and Strahorn (4, p. 129) claim that "the soils of the Northport, Washington, area are seriously impaired by the excessive leaching of the acid soluble components due to SO_2 from Trail smelter, B. C." Although they did not investigate them they claim alterations of the texture and structure of the soil and a decrease in the amount of organic matter

therein by leaching due to the SO_2 fumes from the same source. The Trail smelter is located 12.5 miles north of the International boundary along the winding course of the Columbia River, or about 6 miles directly north of it.

On the other hand, Katz, Wyatt, and Atkinson (11, p. 22) conclude from much more extensive data that "the soils in Canada in the vicinity of Trail are being affected by the presence of smelter fumes. The effect consists of marked increase in the acidity of the soil, a decrease in the ex-changeable bases of the soil, and an increase in sulphate content. This effect diminishes quite rapidly with increasing distance from Trail and becomes negligible in the region between Columbia Gardens and the International boundary south of Trail. Within the Northport area none of the soils can be considered as showing the Trail effect with the exception of the limited area of mission soil near the Zurawski place which is situated close to the International boundary."

Crocker (7) testified on the basis of sulphate determinations in rain-water at Northport, Washington, by Griffin and Potter that less than 8 pounds of sulphur per acre year is washed down at this place by precipitation. He also testified that this is much less than is washed down by precipitation in many good agricultural regions in the United States and cited Ames, Iowa, with 15 pounds of sulphur per acre year, Liberty, Indiana, with 46 to more than 54 pounds, and Urbana, Illinois, with 40 to 45 pounds per acre year.

Finally, attention should be called to the losses of lime from soils as indicated by the amounts to apply as recommended by authorities in the United States. According to Weir (20, p. 324) an acre of strongly acid sandy loam requires about one ton of finely powdered limestone to change its reaction 1 pH to a depth of five or six inches. More strongly buffered soils are reported to respond to two tons per acre for the production of general farm crops. After the establishment of the proper reaction he reports the use of 1000 pounds per acre once in a four to six-year period in the older agricultural sections.

Lyon and Buckman (14, p. 275) suggest a range of 1500 to 2500 pounds per acre of finely ground limestone be applied to sandy loam and from 2000 to 3500 pounds to clay loam in a rotation in which red clover is grown.

Brown and Munsell (2, p. 275) estimate "that about 300 pounds of limestone per acre annually will maintain a given pH in the plow layer of similar soils not receiving physiologically acid fertilizer." The soil employed in their studies was Charlton fine sandy loam.

It appears, therefore, that it would require the deposition of large amounts of sulphur from the air to displace as much calcium from soils as that removed through cropping and leaching under normal conditions in the humid regions.

There is also the reaction of rain water to be considered. MacIntire and Young (15, p. 225) in their studies on the composition of rainfall at ten widely separated locations in Tennessee found all of the 320 duplicate titrations to be alkaline toward methyl orange and with three exceptions where cochineal was employed as the indicator. In commenting on their results they state "the consistent results upon the reaction of rain waters at different points and the excess of soluble bases over soluble acid radicals indicate that soils are not more depleted of bases, nor made more acid by rainfall than they would be if the precipitation were pure water. There is certainly just as much justification for the assumptions that the rainfall components are beneficial, as that to the contrary." They also point out that most of the soluble sulphates found in rain waters have been dissolved from solid matter and that the volume of such discharged into the atmosphere is the primary factor governing the concentration of sulphates in the rain water. They also cite the results of Crowther *et al.* near Leeds. Notably, "of 153 samples collected at seven different points, 140 were either neutral or distinctly alkaline to methyl orange, congo red, and litmus, while the remaining 13 samples were acid." Furthermore, smoke which arises from the burning of coal "may in some cases contain its sulfur not mainly as sulfur dioxide, but almost entirely in the form of sulfate."

In addition it may be that the suspended matter in the atmosphere, such as soil colloids and limestone dust from different sources, reacts with the acid gas to form sulphates. If so, the sulphate content of the precipitation does not necessarily indicate that the soil would be depleted of bases to the theoretical extent.

Pearson *et al.* (18) recently reported results of studies on the pH values of rain water. The results are as follows:

	Apr.	*July*	*Oct.*	*Jan.*
Burnley Town Hall	3.0	4.0	3.0	3.0
Edinburgh, Princes Street	6.0	5.4	6.3	5.3
Liverpool, Netherfield Road	4.4	5.8	4.4	3.7
London, Horseferry Road	6.5	8.0	6.0	4.0
" Kew, N.	5.8	6.0	4.9	5.6
" Garston, Watford	5.2	4.5	4.0	4.5
Rochdale Electric Works	4.7	5.0	5.1	4.6
Southport, Hesketh Park	5.2	5.2	4.9	4.8

Determinations of pH of 25 samples of rain and snow collected in porcelain pans at the Boyce Thompson Institute from October 1937 to March 1938 show a mean of 4.55 with an average deviation of ±0.55 pH. The maximum pH obtained by the quinhydrone electrode was 6.58, the minimum 3.44. Titration curves obtained with 0.01 N HCl and 0.01 N NaOH revealed the samples to be only slightly buffered. The results are similar to those reported by Griffin and Potter from Northport, Washington.

RESULTS

The data for the averages of pH values, titratable acidity, base exchange, and sulphur content were analyzed statistically. The regression coefficients on distance from the city halls and significance are presented in Table II. Scatter diagrams and regression lines are given in Figures 1 to 8, inclusive.

It is to be noted from Figure 1 that the titratable acidity in Yazoo clay varies significantly with distance from the city hall of East St. Louis. The lowest pH values for this soil type, however, were those collected within a distance of about 1000 feet from a chemical plant (soil 36) and a round house (soil 37). The other soil characteristics studied did not show such trends.

According to Figure 2 only the regression line for sulphur content of Memphis silt loam was significant. There was no relationship between distance of sampling from the city hall of St. Louis, Missouri, and the amount of exchangeable calcium magnesium and the physical composition of this soil.

As revealed by Figure 3, the regression lines for pH values, titratable acidity, base exchange capacity, and the sulphur content of Clinton silt loam are not significant. It should be noted also that the amount of sand, silt, and clay does not vary with distance of sampling from the city hall of East St. Louis.

The Yazoo loam and sarpy fine sandy loam samples were taken from 0 to 8 inches. The pH values of the former were 3.0 miles northeast, 3.5 miles northeast, 3.5 miles north, 5.5 miles north, 8 miles south, and 15 miles north of East St. Louis city hall were 4.78, 5.94, 5.06, 5.79, 5.55, and 5.35 respectively. The samples having the lowest values were taken from an area adjacent to a chemical plant. These samples also had the highest titratable acidity, but the differences between the base exchange capacity and physical composition of these samples and the others were insignificant.

The sarpy fine sandy loam samples were alkaline in reaction. The pH values were 7.34, 1 mile south; 7.03, 2.5 miles south; 7.14, 3 miles south; 7.47, 4 miles north; 7.60, 9 miles north; and 7.33, 10 miles north of the city hall. The variations recorded in the titratable acidity base exchange capacity and the amount of sand, silt, and clay in the samples were remarkably narrower in range.

The regression lines for pH values, titratable acidity, and base exchange capacity of the soils collected in the Camden and Philadelphia areas, or sassafras sand (Fig. 4), Collington sand (Fig. 5), Collington fine sandy loam (Fig. 6), and sassafras loam (Fig. 7) are not significant.

TABLE II

REGRESSION COEFFICIENTS ON DISTANCE AND SIGNIFICANCE OF pH, TITRATABLE ACIDITY, BASE EXCHANGE, AND SULPHUR

Soil type	pH value		Titratable acidity		Base exchange capacity		Sulphur	
	Regression coefficient on distance	Significance*	Regression coefficient on distance	Significance*	Regression coefficient on distance	Significance*	Regression coefficient on distance	Significance*
Yazoo clay	0.044	0.05–0.02	3.483	0.01	0.01244	0.9	0.00157	0.6 –0.5
Clinton silt loam	0.0095	0.7 –0.6	0.1704	0.6–0.5	0.0293	0.9	0.000479	0.6 –0.5
Memphis silt loam	0.062	0.2 –0.1	0.477	0.4–0.3	0.148	0.2–0.1	0.00183	0.02–0.01
Collington sand	0.009484	0.2 –0.1	0.017	0.9–0.8	0.0072	0.9–0.8		
Collington fine sandy loam	0.02993	0.05–0.02	0.24897	0.5–0.4	0.06332	0.5–0.4		
Sassafras sand	0.015465	0.4 –0.3	0.6418	0.2–0.1	0.0717	0.4–0.3		
Sassafras loam	0.0395	0.5 –0.4	0.3662	0.5–0.4	0.0062	0.9		

* Under the column headed Significance is shown the probability of obtaining the ratio regression coefficient/standard deviation by chance. Unless this probability was less than 0.02, the coefficient was not considered significant.
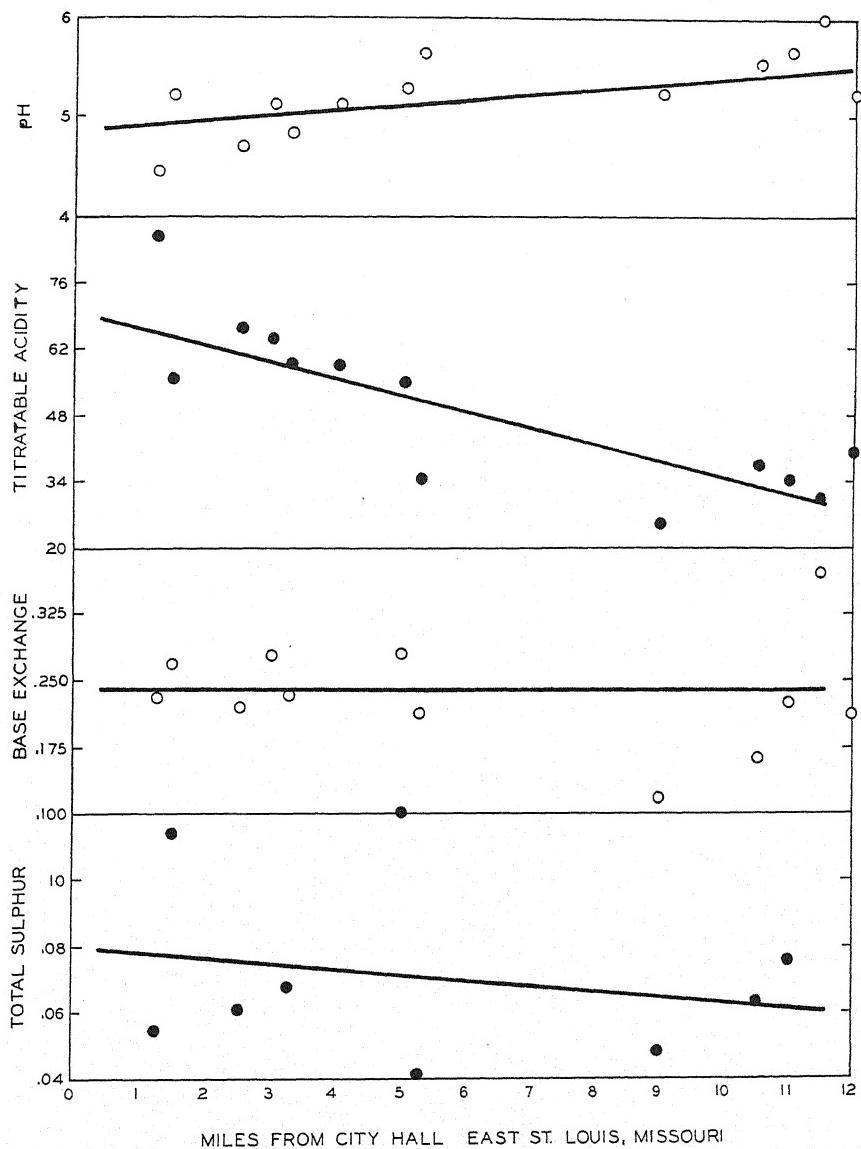
FIGURE 1. Yazoo clay showing pH values, titratable acidity, base exchange, and total sulphur at varying distances from city hall of East St. Louis, Missouri.
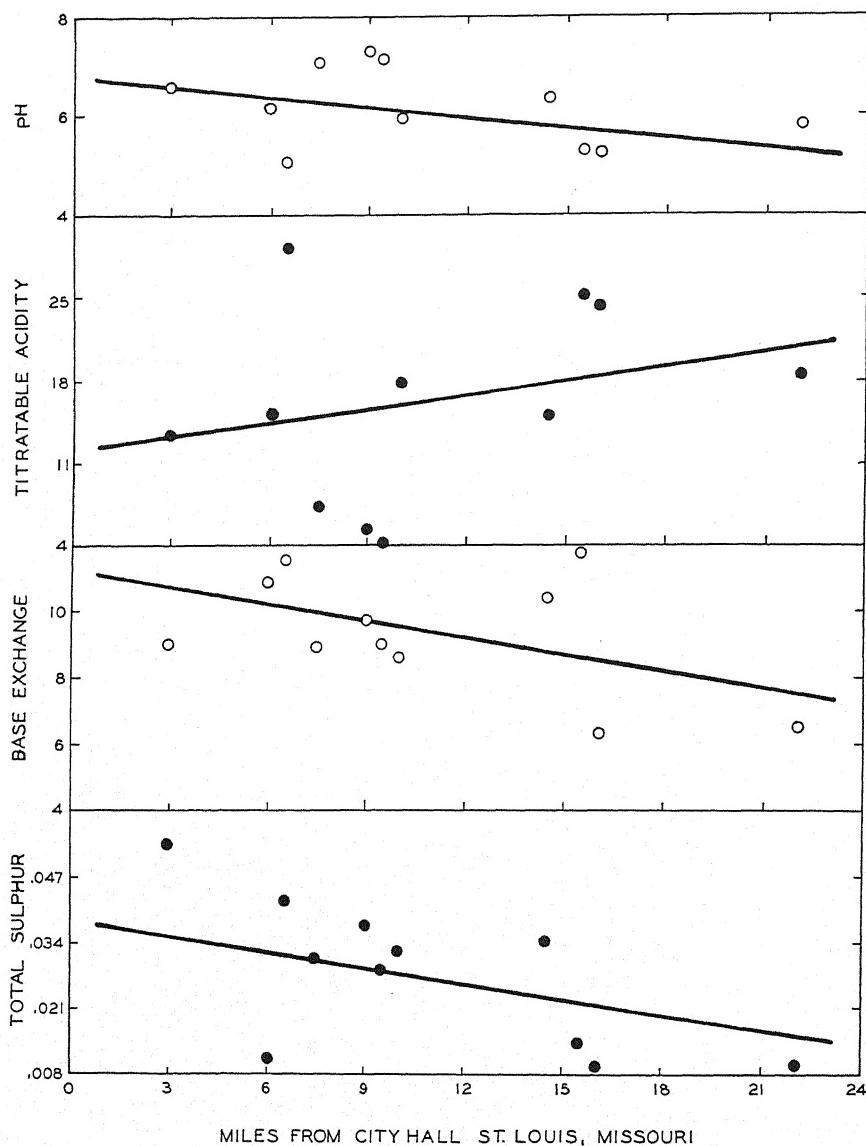
FIGURE 2. Memphis silt loam showing pH values, titratable acidity, base exchange, and total sulphur at varying distances from city hall of St. Louis, Missouri.
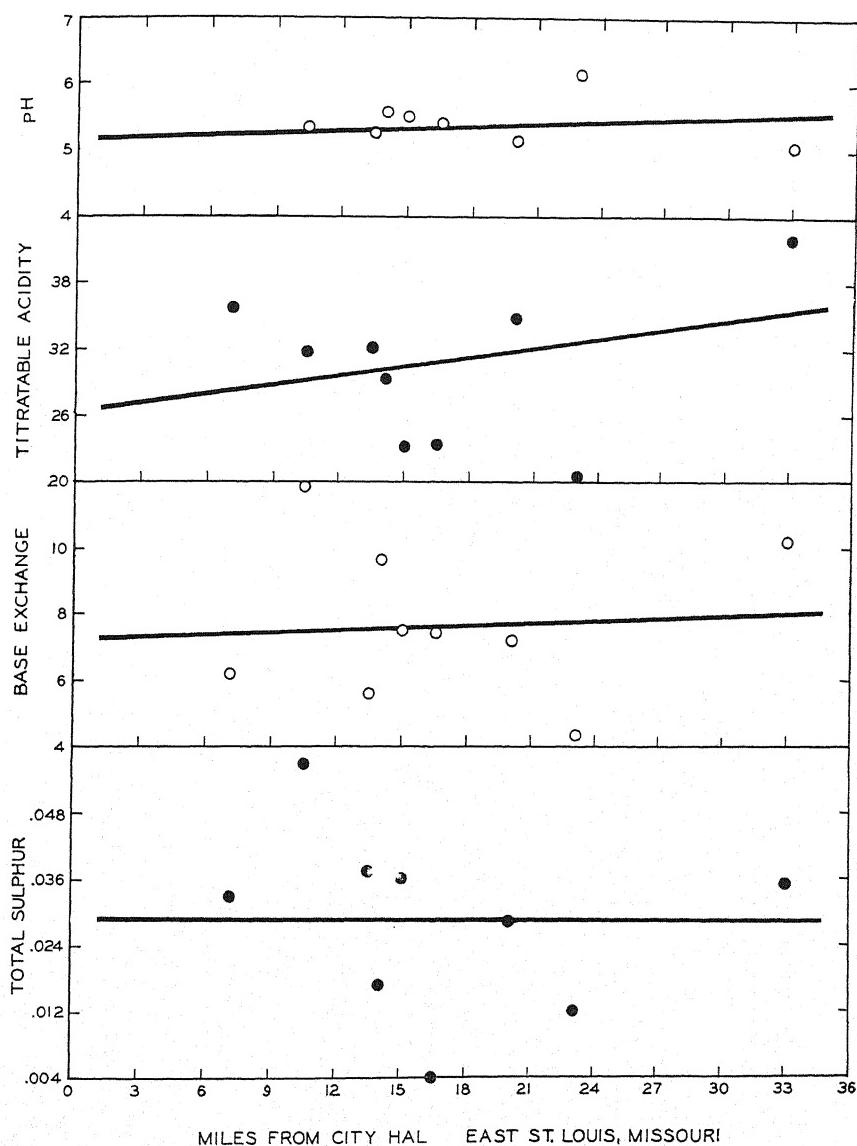
FIGURE 3. Clinton silt loam showing pH values, titratable acidity, base exchange, and total sulphur at varying distances from city hall of East St. Louis, Missouri.
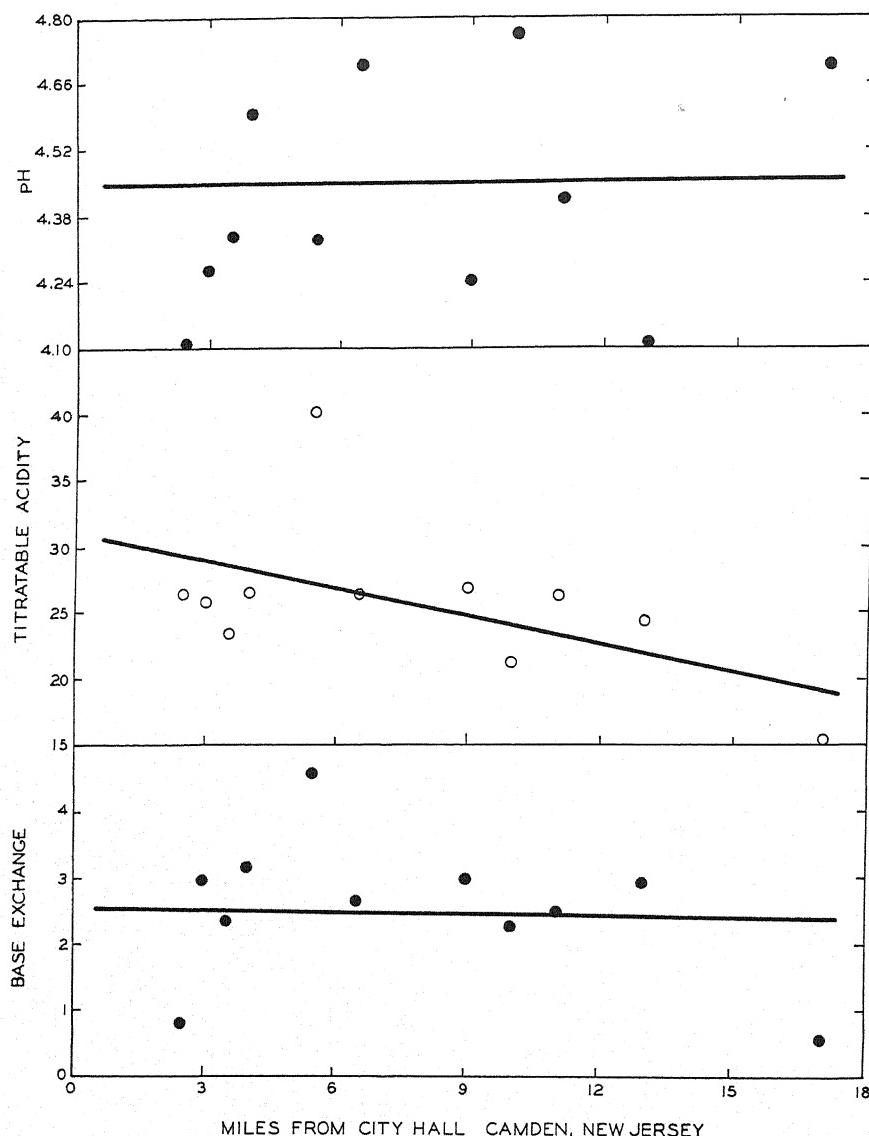
FIGURE 4. Sassafras sand showing pH values, titratable acidity, and base exchange at varying distances from city hall of Camden, New Jersey.
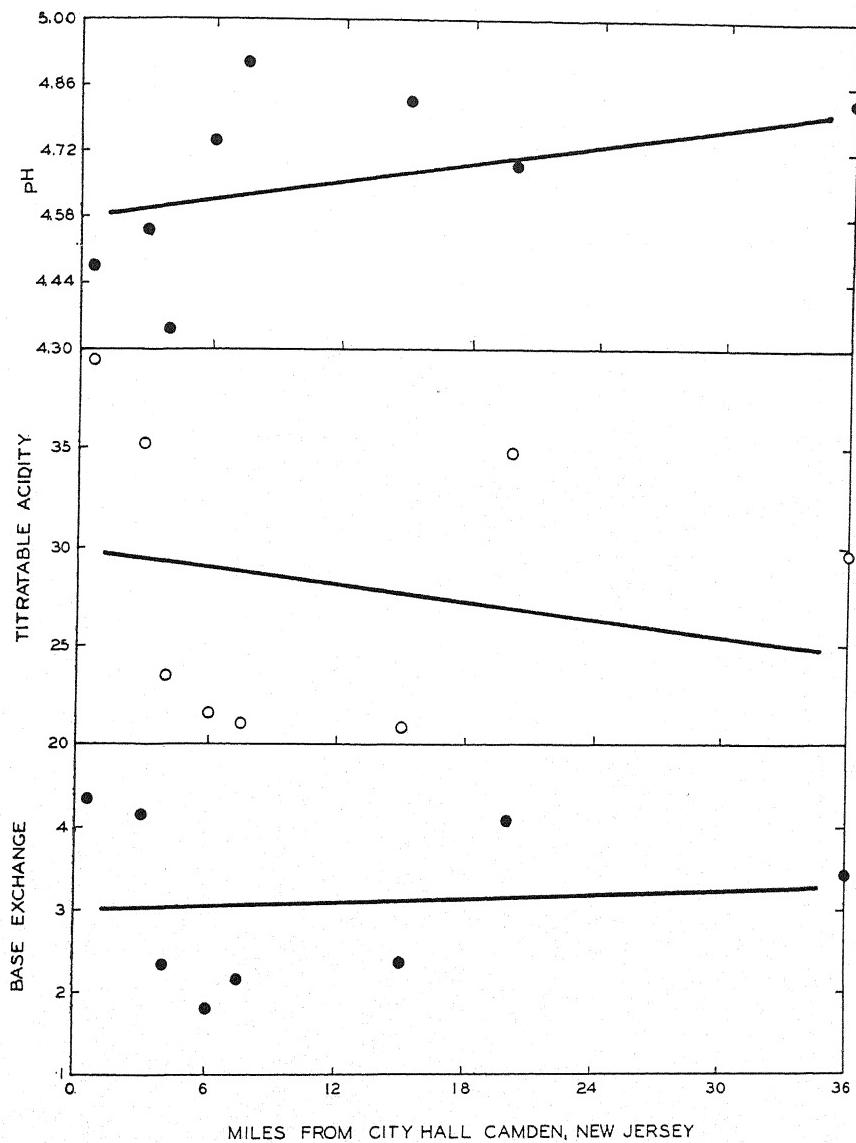
FIGURE 5. Collington sand showing pH values, titratable acidity, and base exchange at varying distances from city hall of Camden, New Jersey.
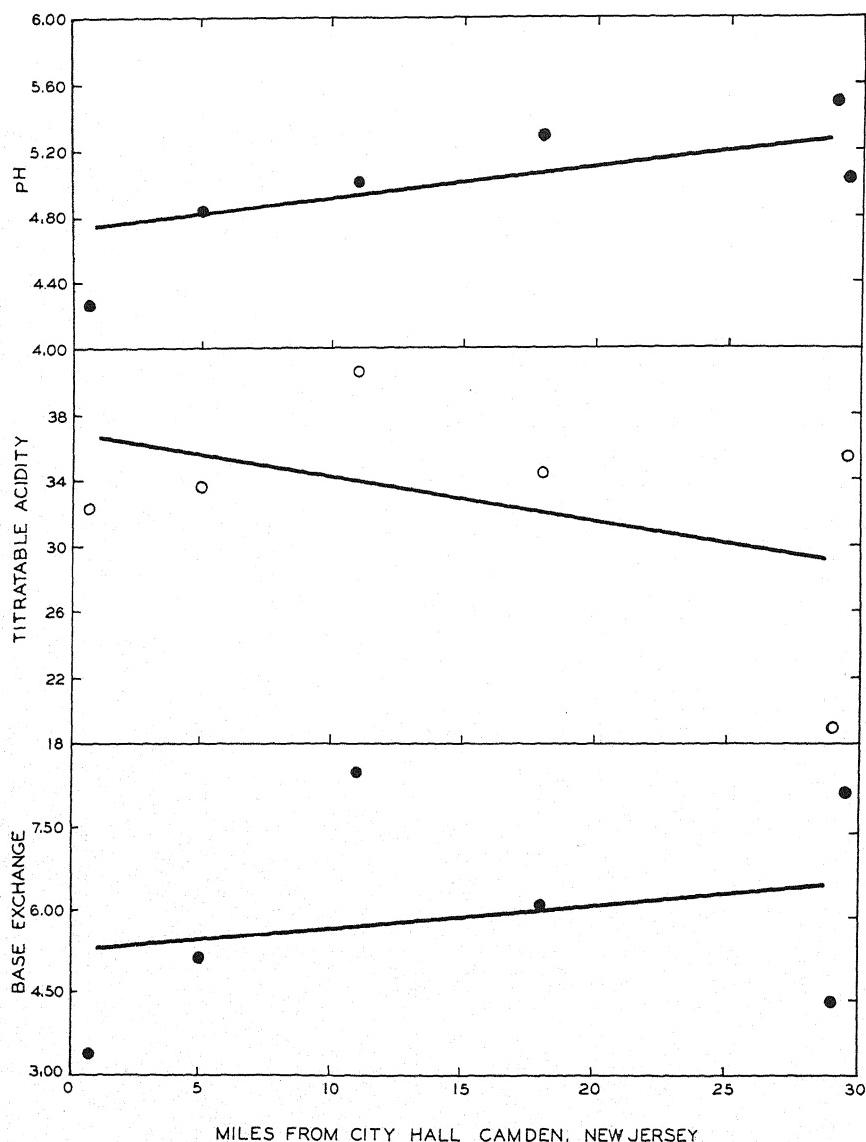
FIGURE 6. Collington fine sandy loam showing pH values, titratable acidity, and base exchange at varying distances from city hall of Camden, New Jersey.
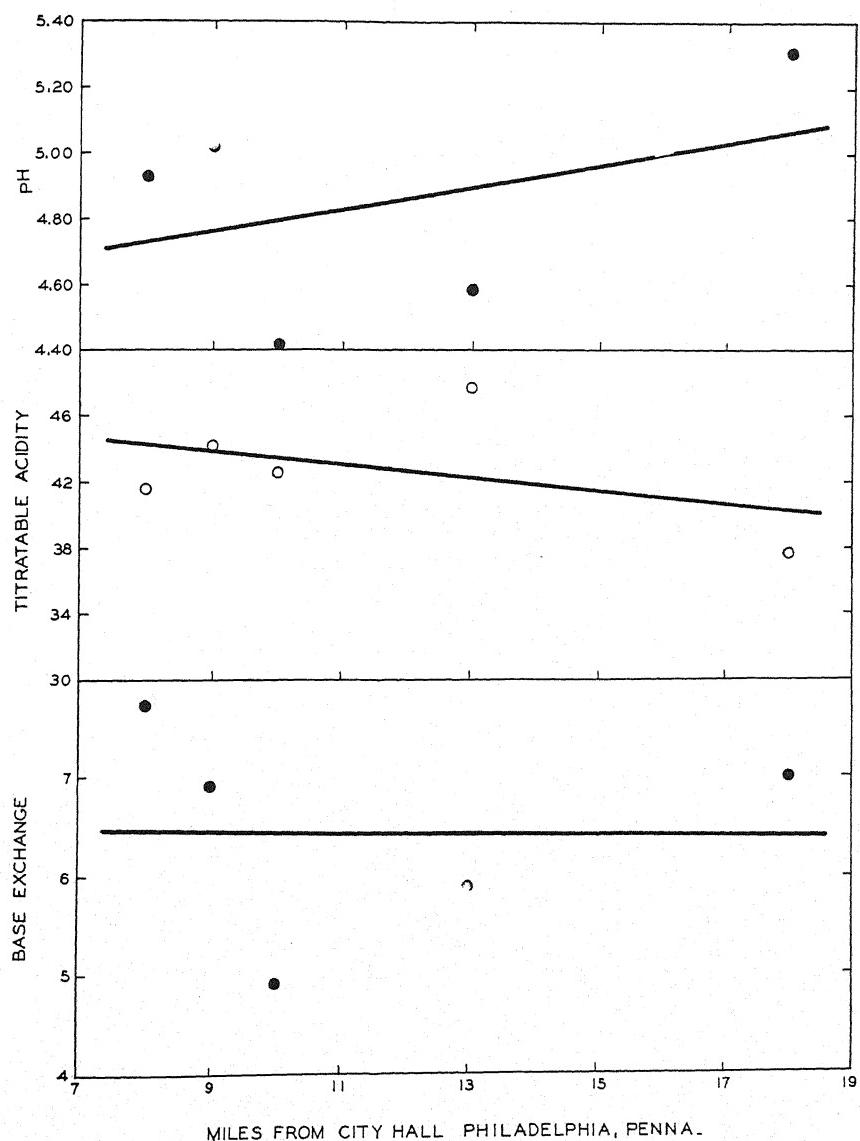
FIGURE 7. Sassafras loam showing pH values, titratable acidity, and base exchange at varying distances from city hall of Philadelphia, Pennsylvania.

SUMMARY AND CONCLUSIONS

Many samples of sassafras sand, Collington sand, and Collington fine sandy loam were collected at varying distances from the city halls of Camden, New Jersey, sassafras loam from Philadelphia, Pennsylvania, Yazoo clay, Yazoo loam, sarpy fine sandy loam, and Clinton silt loam from East St. Louis, Illinois, and Memphis silt loam from St. Louis, Missouri.

The pH values, titratable acidity, base exchange capacity, replaceable calcium and magnesium, sulphur content, the percentage of sand, silt, and clay were determined. With the exception of the titratable acidity in Yazoo clay and the sulphur content of the Memphis silt loam, statistical analysis of the data did not show a significant trend with distance in the results obtained from a given soil type. These natural fumigations of SO_2 of the order of magnitude studied, caused no appreciable alteration in the soil characteristics.

It is evident from these results that soils about centers of considerable sulphur dioxide production, like St. Louis, Missouri, have not been noticeably altered in spite of many years of exposure. The slight acidulation, if any, is much less than the acidulation due to natural processes in the soil and could be overcome by slight increases in the rate of lime application.

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NITROGEN AND SULPHUR CONTENT OF LEAVES OF PLANTS WITHIN AND AT DIFFERENT DISTANCES FROM INDUSTRIAL CENTERS

M. M. McCool and Arnold N. Johnson

In addition to the investigations on soil characteristics in the Camden, New Jersey, East St. Louis, Illinois, and St. Louis, Missouri areas (3) we have made observations on the growth conditions of the vegetation in them. Samples of several plant species grown on several of the stations located at different distances from the centers of the cities have been collected and the percentage of nitrogen, total sulphur, and sulphate sulphur have been determined.

There are many reports on the sulphur content of the vegetation of areas adjacent to sources of sulphur dioxide, but there are only a few which have attempted to determine the decrease in sulphur content of the vegetation with increase of distance from the source of the SO_2.

Fisher *et al.* (1) review the literature which bears on the fixation of sulphur dioxide and present average results of analyses of several species of plants. They report that sulphur dioxide polluted atmosphere increased the sulphur content of vegetation in the upper Columbia River valley through a distance of about 54 miles from the Trail, B.C., smelter.

Katz and McCallum (2) studied the sulphur content of larch, hazel, ninebark, aspen, Douglas fir, and yellow pine in 1930, 1931, and 1934, in the upper Columbia River valley. In 1935 the two latter only were included in their investigations. They found that Douglas fir proved to be a more sensitive indicator of the distribution of sulphur dioxide than yellow pine and reported increases in the sulphur content of its leaves 57 miles from the Trail smelter. In addition they found conifer needles to absorb sulphur dioxide only during the growing season and in addition the sulphur content of needles increases from year to year in regions where sulphur dioxide is present in the atmosphere. They show that the total sulphur and sulphate sulphur content of plants may be increased greatly without injury.

METHODS OF PROCEDURE

Specimens of goldenrod (*Solidago* sp., *S. microphylla* Engelm.) referred to as A and B, respectively, in tables and text, milkweed, small and large (*Asclepias* sp. and *A. syriaca* L., respectively), yarrow (*Achillea millefolium* L.), large tickle grass (*Agrostis* sp.), ragweed (*Ambrosia artemisiaefolia* L.), smartweed (*Polygonum acre* HBK), dewberry (*Rubus* sp.), dwarf sumach (*Rhus copallina* L.), yellow locust (*Robinia pseudoacacia* L.), and cork elm

TABLE I

REGRESSION COEFFICIENTS OF TOTAL SULPHUR AND SULPHATE SULPHUR CONTENTS ON DISTANCE IN THE LEAVES OF PLANTS GROWN AT VARYING DISTANCES FROM CITY HALLS

Soil type	Kind of plant	Total sulphur		Sulphate sulphur	
		Regression coefficient	Odds of significance*	Regression coefficient	Odds of significance*
	Table II				
Collington sand	Yarrow	−0.0002743		−0.001732	
	Dewberry	−0.000607		−0.000513	
Collington fine sandy loam	Goldenrod A	−0.00998		−0.00623	
	Goldenrod B	−0.002378		−0.0020279	>50:1
	Yarrow	−0.0035195		−0.001494	
	Table III				
	Goldenrod B	−0.00108		−0.00148	
Collington sand	Ragweed	−0.00239		+0.00105	
	Tickle grass (large)	−0.00771		−0.00448	
Collington fine sandy loam	Goldenrod B	−0.00216		−0.00187	
	Ragweed	−0.00448		−0.00467	
	Milkweed (large)	−0.00387			
Sassafras sand	Tickle grass (large)	+0.00866			
	Goldenrod A	−0.0243			
	Goldenrod B	+0.00385			
	Table IV				
	Goldenrod A	−0.0194	>50:1	−0.00677	>50:1
Sassafras sand	Goldenrod B	−0.0149	>50:1	−0.01246	>20:1
	Ragweed	−0.02043	>50:1	−0.0236	>20:1
	Tickle grass (large)	−0.0205	>50:1	−0.01735	>20:1
	Table V				
	Sumach	−0.0133	>50:1	−0.00832	>20:1
Memphis silt loam	Locust	+0.0159		−0.0051	
	Goldenrod B	−0.01004	>50:1	−0.0076	>20:1
	Elm	−0.00569	>20:1	−0.00112	
	Table VI				
	Smartweed	−0.03572	>50:1	−0.0332	>50:1
Yazoo clay	Goldenrod B	−0.0215		−0.0186	
	Ragweed	−0.0237		−0.0252	>20:1
	Milkweed (small)	+0.0132		+0.0242	
Yazoo loam	Smartweed	−0.0231		−0.01295	
	Table VII				
	Goldenrod B	−0.00125		Insufficient data	
Clinton silt loam	Locust	−0.0011		−0.0017	
	Sumach	−0.0026		−0.0019	
	Cherry	−0.00062		−0.000133	
	Table VIII				
Yazoo clay, Illinois	Smartweed	−0.00369		−0.004	
	Milkweed (small)	−0.00397		−0.00414	
Yazoo loam, Illinois	Smartweed	−0.00891		−0.00629	

* Odds less than 20:1 unless otherwise stated.

(*Ulmus racemosa* Thomas) were collected during the summers of 1936 and 1937 at several different stations located on certain soil types and at varying distances from the city halls of Camden, New Jersey, East St. Louis, Illinois, and St. Louis, Missouri, respectively as previously reported (3). It should be noted that not all of the above species of plants were present on each station. Leaves were removed from three or more elm, locust, and cherry trees and twenty-five or more of each of the other species. The specimens were subjected to air-drying, after which the leaves were removed and ground to a fine powder by means of a power-driven grinder.

TABLE II

SULPHUR AND SULPHATE SULPHUR CONTENT (PER CENT DRY WEIGHT BASIS) OF LEAVES OF PLANTS TAKEN FROM CAMDEN, NEW JERSEY AREA JULY 7 TO 10, 1936

Soil type	Collington sand				Collington fine sandy loam			
Station number Mileage* Direction*	6 0.5 ESE	8 7.5 SSE	3 15 E	12 36 NE	9 0.7 E	7 5 SE	4a 18 E	5 29 ENE
Goldenrod A Sulphur Sulphates					0.525 0.214	0.306 0.096	0.213 0.023	0.194 0.016
Goldenrod B Sulphur Sulphates					0.228 0.055	0.204 0.048	0.156 0.015	0.163 0.000
Yarrow Sulphur Sulphates	0.274 0.208	0.178 0.064	0.200 0.057	0.233 0.103	0.312 0.155	0.245 0.098	0.217 0.095	0.196 0.097
Dewberry Sulphur Sulphates	0.155 0.035	0.131 0.023	0.125 0.022	0.127 0.014				

* From city hall.

The Parr bomb method of procedure was followed in making total sulphur determinations. The sulphates were removed by shaking three hours with dilute hydrochloric acid (6 ml. concentrated HCl in 500 ml. distilled water) and allowing to stand overnight. The standard Kjeldahl method modified to include nitrates was employed in nitrogen determinations. Careful observations were made during each visit as to sulphur dioxide injury to the vegetation in the areas under consideration. Numerous photographs were taken in the East St. Louis, Illinois and St. Louis, Missouri areas by L. P. Flory, photographer for the Boyce Thompson Institute.

The dates on which the areas were visited were as follows: Camden, New Jersey area—July 7 to 10, August 15 to 17, and September 2 to 5, 1936; April 26 to 28, June 10 to 12, and August 6 to 8, 1937; East St. Louis and St. Louis areas—July 25 to 30, and October 8 to 16, 1936; May 4 to 10, May 25, to 27, and June 10 to 16, 1937.

TABLE III

SULPHUR AND SULPHATE SULPHUR (PER CENT DRY WEIGHT BASIS) OF LEAVES OF PLANTS FROM CAMDEN, NEW JERSEY AREA

Soil type	Taken September 2 to 5, 1936 Collington sand							Taken June 10 to 12, 1937 Collington fine sandy loam					Taken June 10 to 12, 1937 Sassafras sand				
Station number	6	6I	7I	8	3	60	12	9	7	4a	5	58	54	51	52	55a	53a
Mileage*	0.5	3	4	7.5	15	20	36	0.7	5	18	29	29	2.5	3	6.5	6.5	11
Direction*	ENE	SE	E	SSE	E	E	NE	E	SE	E	ENE	NE	S	NE	ENE	S	NE
Goldenrod B																	
Sulphur	0.225	—	0.252	—	0.228	0.222	0.199	0.286	0.175	0.199	0.166	0.199	0.150	0.242	0.130	0.175	0.237
Sulphates	0.089	—	0.075	—	0.030	0.016	0.042	0.099	0.015	0.023	0.026	0.004					
Ragweed																	
Sulphur	0.762	—	—	0.504	0.628	0.571	0.604	0.873	0.676	0.591	0.645	0.715					
Sulphates	0.495	—	—	0.262	0.497	0.366	0.464	0.521	0.389	0.321	0.412	0.281					
Tickle grass (large)																	
Sulphur	0.370	0.496	0.411	—	0.302	0.291	—						0.245	0.270	0.247	0.328	0.328
Sulphates	0.157	0.199	0.080	—	0.078	0.079	—										

* From city hall.

RESULTS

The vegetation growing in the areas under observation was examined carefully during each visit for sulphur dioxide injury but none was found.

The data obtained from total sulphur and sulphate sulphur determinations were analyzed statistically with respect to distance of sampling from the various city halls. The regression coefficients on distance and odds of significance are summarized in Table I.

The variations in the percentage of total sulphur in the leaves of yarrow and dewberry plants grown on Collington sand, Camden, New Jersey area, sampled July 7 to 10, 1936, are not significant as revealed by an

TABLE IV

Sulphur and Sulphate Sulphur Content (Per Cent Dry Weight Basis) of Leaves of Plants Taken from Camden, New Jersey Area August 15 to 17, 1936

Soil type	Sassafras sand						
Station number Mileage* Direction*	51 2.3 NE	54 4 S	52 5.5 ENE	53 6.5 NE	55a 6.5 S	53a 11 NE	56 12 SE
Goldenrod A							
Sulphur	0.427	—	0.311	0.336	0.312	—	0.228
Sulphates	0.068	—	0.035	0.038	0.031	—	0.000
Goldenrod B							
Sulphur	0.312	—	0.243	0.259	0.230	—	0.163
Sulphates	0.176	—	0.107	0.118	0.084	—	0.049
Ragweed							
Sulphur	0.825	0.777	0.789	0.785	0.788	0.645	0.635
Sulphates	0.579	0.507	0.660	0.564	0.515	0.407	0.354
Tickle grass (large)							
Sulphur	0.474	0.446	0.381	0.418	0.394	0.371	0.213
Sulphates	0.239	0.262	0.149	0.205	0.192	0.172	0.027

* From city hall.

examination of Tables I and II. It should be noted, however, that the sulphate sulphur in goldenrod B, taken from Collington fine sandy loam, decreases significantly with the increase in distance from the city hall.

Additional plant samples were collected from Collington sand September 2 and 3, 1936, Collington fine sandy loam and sassafras sand June 10 to 12, 1937. As shown by the regression coefficients and odds of significance in Table I and the data in Table III, the variations in the total sulphur and sulphate sulphur in the plants sampled are not significant with respect to the distance from the city hall.

Plants growing on sassafras sand were collected August 15 to 17, 1936. As the data in Tables I and IV reveal the variations in the total sulphur and sulphate sulphur content of goldenrod A, total sulphur of goldenrod

TABLE V

SULPHUR AND SULPHATE SULPHUR CONTENT (PER CENT DRY WEIGHT BASIS) OF LEAVES OF PLANTS TAKEN FROM ST. LOUIS, MISSOURI, JULY 25 TO 30, 1936

Soil type	Memphis silt loam											
Station number	27	23	12	13	24	14	25	15	16	26	17	18
Mileage*	3	6	6.5	7.5	9	9.5	10	10.5	12	14.5	16	22
Direction*	S	SW	N	N	SW	N	S	N	NW	S	W	W
Sumach												
Sulphur	—	—	0.353	0.279	0.297	0.303	0.168	0.191	0.191	0.158	0.133	0.142
Sulphates	—	—	0.185	0.087	0.158	—	0.041	—	0.038	0.041	0.026	0.033
Locust												
Sulphur	0.421	—	—	—	—	0.242	—	0.283	—	0.239	0.268	0.219
Sulphates	0.154	—	—	—	—	0.031	—	0.014	—	0.017	0.000	0.000
Goldenrod B												
Sulphur	0.358	0.324	0.311	0.215	0.268	—	0.187	—	0.196	0.185	0.183	0.173
Sulphates	0.121	0.143	0.170	0.004	0.055	—	0.007	—	0.063	0.011	0.021	0.000
Elm												
Sulphur	0.261	0.241	0.152	—	—	0.139	0.143	0.140	0.138	0.139	0.133	0.142
Sulphates	0.086	0.083	0.036	—	—	0.032	0.037	0.018	0.012	0.019	0.017	0.081

* From city hall.

TABLE VI

SULPHUR AND SULPHATE SULPHUR CONTENT (PER CENT DRY WEIGHT BASIS) OF LEAVES OF PLANTS TAKEN FROM EAST ST. LOUIS, ILLINOIS, JULY 25 TO 30, 1936

Soil type	Yazoo clay												Yazoo loam			
Station number	36	28	46	2	31	37	32	30	35	5	38	34	1	3	4	33
Mileage*	1.25	1.5	2	2	2.25	2.5	5	6	6	6.5	7	11	3	3.5	8	15
Direction*	E	E	SE	E	E	E	N	E	NE	S	E	NNE	E	S	S	N
Smartweed																
Sulphur	0.612	0.452	0.530	0.465	0.492	0.569	0.427	0.302	0.299	0.275	0.262	0.282	0.655	0.298	0.357	0.217
Sulphates	0.428	0.295	0.341	0.314	0.327	0.326	0.243	0.119	0.182	0.133	0.119	0.107	0.404	0.143	0.171	0.133
Goldenrod B																
Sulphur	0.480	—	—	0.307	0.364	—	0.335	—	—	—	0.207	0.220				
Sulphates	0.301	—	—	0.137	0.124	—	0.143	—	—	—	0.023	0.056				
Ragweed																
Sulphur	0.920	0.869	—	0.861	0.741	—	0.702	0.689	0.588	—	0.581	0.742				
Sulphates	0.638	0.582	—	0.562	0.410	—	0.443	0.395	0.334	—	0.319	0.401				
Milkweed (small)																
Sulphur	—	—	—	0.917	—	—	—	0.961	1.287	—	0.803	1.086				
Sulphates	—	—	—	0.667	—	—	—	0.770	0.822	—	0.644	0.909				

* From city hall.

B, ragweed, and tickle grass with distance from city hall are significant. The trend for sulphate sulphur content of goldenrod B, ragweed, and tickle grass are in the same direction.

Specimens of sumach, locust, goldenrod B, and elm growing on Memphis silt loam in the St. Louis, Missouri area were collected July 25 to 30, 1936. The results of the chemical analyses are presented in Table V and the statistical analyses in Table I. According to these results, the total sulphur in sumach and goldenrod B varied significantly with the distance from the city hall and the total sulphur in the leaves of elm and the sulphate sulphur in those of sumach and goldenrod B were in this direction.

TABLE VII

SULPHUR AND SULPHATE SULPHUR CONTENT (PER CENT DRY WEIGHT BASIS) OF LEAVES OF PLANTS TAKEN FROM EAST ST. LOUIS, ILLINOIS, JULY 25 TO 30, 1936

Soil type	Clinton silt loam						
Station number Mileage* Direction*	29 6 ESE	39 10 NE	6 10.5 SE	40 14 NE	7 15 SE	9 23 SE	43 33 NE

Goldenrod B
  Sulphur
  Sulphates
	0.211
	0.017
		0.199
		0.000
			0.193
			0.000
				0.226
				0.000
					0.183
					0.000
						0.153
						0.000
							0.188
							0.000

Locust
  Sulphur
  Sulphates
	—
	—
		0.338
		0.058
			—
			—
				—
				—
					0.246
					0.028
						0.237
						0.010
							0.296
							0.016

Sumach
  Sulphur
  Sulphates
	0.238
	0.117
		0.378
		0.205
			0.212
			0.084
				0.192
				0.087
					—
					—
						0.210
						0.075
							0.207
							0.093

Cherry
  Sulphur
  Sulphates
	0.118
	0.013
		0.153
		0.015
			—
			—
				—
				—
					—
					—
						0.096
						0.010
							0.125
							0.011

* From city hall.

The results of the chemical analyses of the leaves of plants removed from Yazoo clay and Yazoo loam in the East St. Louis, Illinois area July 25, to 30, 1936 are presented in Table VI and the statistical summary in Table I. According to the latter the regression coefficients on distance for total sulphur and sulphate sulphur in smartweed grown on Yazoo clay are significant, and there is the same trend in the sulphate sulphur in ragweed. There are no trends for these in goldenrod B, ragweed, and smartweed removed from Yazoo loam.

Samples of goldenrod B, locust, sumach, and cherry were collected from Clinton silt loam in the East St. Louis area on the above dates. According to the statistical analyses (Table I) and the results of analyses presented in Table VII the variation in the sulphur and sulphate sulphur

with distance from the city hall were not significant. In no case were the odds greater than 20:1.

Smartweed and milkweed (small) were collected from Yazoo clay and the former from Yazoo loam June 10 and 11, 1937, and the usual determinations made. It is to be noted from Table I that the results obtained from these early samplings are not significant. According to the results in Table VIII the sulphur and sulphate sulphur content, however, were highest in those samples which were in close proximity to the city hall.

TABLE VIII

SULPHUR AND SULPHATE SULPHUR CONTENT (PER CENT DRY WEIGHT BASIS) OF LEAVES OF PLANTS TAKEN FROM EAST ST. LOUIS, ILLINOIS AREA JUNE 10 TO 11, 1937

Soil type	Yazoo clay, Illinois						Yazoo loam, Illinois			
Station number	80	71	74	75	82	83	51	1	69	73
Mileage*	3	8	33	35	41	42	1.75	3	7	24
Direction*	E	NE	S	S	S	S	S	NE	N	S
Smartweed										
Sulphur	0.352	0.221	0.176	0.204			0.451	0.373	0.315	0.215
Sulphates	0.250	0.136	0.115	0.062			0.371	0.187	0.139	0.136
Milkweed (small)										
Sulphur	0.705	0.525	0.566	0.273	0.491	0.577				
Sulphates	0.521	0.325	0.376	0.127	0.318	0.328				

* From city hall.

Many additional samples were analyzed. Since they represent only two or three stations they are not presented. It should be noted, however, that the highest sulphur and sulphate sulphur contents, with few exceptions, were found to be in those within or in close proximity to the various city limits.

The nitrogen content of all samples collected was ascertained. Although the results are not given, attention should be called to the fact that there was no relationship between the total sulphur content and the amount of nitrogen in the leaves. High nitrogen values were often associated with high and low total sulphur contents.

SUMMARY

It is to be concluded from these studies that the sulphur dioxide from smoke-producing areas did not adversely affect the vegetation in the Camden, New Jersey, East St. Louis, Illinois, and St. Louis, Missouri areas. With few exceptions, the total sulphur and sulphate sulphur contents of the leaves of the plants collected did not decrease significantly with distance from the sources of SO_2. Although comparatively few of the individual regression coefficients differed significantly from zero, the fact that 30 out of 34 with regard to total sulphur, and 27 out of 29 with regard to

sulphate sulphur were negative in sign, makes it quite clear that, on the whole, there is a significant decrease in sulphur content of the plants with increase in distance from the sources of sulphur dioxide.

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X-RAY DIFFRACTION ANALYSIS AND ITS APPLICATION TO THE STUDY OF PLANT CONSTITUENTS[1]

Wayne A. Sisson[2]

The word "X-ray" usually suggests to the layman a radiograph of a decayed tooth or a broken bone; to the botanist it may suggest some of the biological effects of radiation; but to the physicist or chemist it is certain to suggest the phenomenon of diffraction. This latter phase of X-ray work will be discussed in the present paper.

CRYSTAL STRUCTURE AND X-RAY DIFFRACTION

This particular branch of science is based upon the facts, (a) that most solid materials are crystalline, and (b) that X-rays are diffracted in passing through crystalline materials.

By modern definition, a crystalline material is one in which the atoms or molecules are arranged in an orderly fashion. Thus, if atoms were large enough to see, it would be possible to observe several planes of atoms as a sodium chloride crystal is viewed from different angles. If a beam of X-rays is passed through this arrangement a small portion of the beam is diffracted away from the main beam by each one of the set of planes represented in Figure 1 A.

The diffraction pattern shown in Figure 1 B illustrates what happens when X-rays pass through a single crystal of iron. The center spot is the undiffracted beam. Each one of the outer spots is the diffracted ray from a particular family of planes in the crystal lattice.

X-ray diffraction takes place according to a definite law. In Figure 1 C are represented two planes of atoms, with the X-ray beam passing through at angle θ. X-rays are reflected by the top layer, and also by the second layer. Whenever the distance d between these two layers and the angle θ is such that the distance c p b is equal to the wave-length of the X-rays, then the reflected wave at b will be in phase with that at a and they will reinforce each other to produce a diffracted ray. Thus, if the wave-length λ of the X-rays, and the angle θ at which they are diffracted are known, it is possible to calculate the distance d between the two layers of atoms from the equation $n\lambda = 2d\sin\theta$.

EQUIPMENT AND EXPERIMENTAL TECHNIQUE

Figure 2 A is a photograph of the equipment used in making the diffraction patterns shown later. In front is the regulating stand. The high tension

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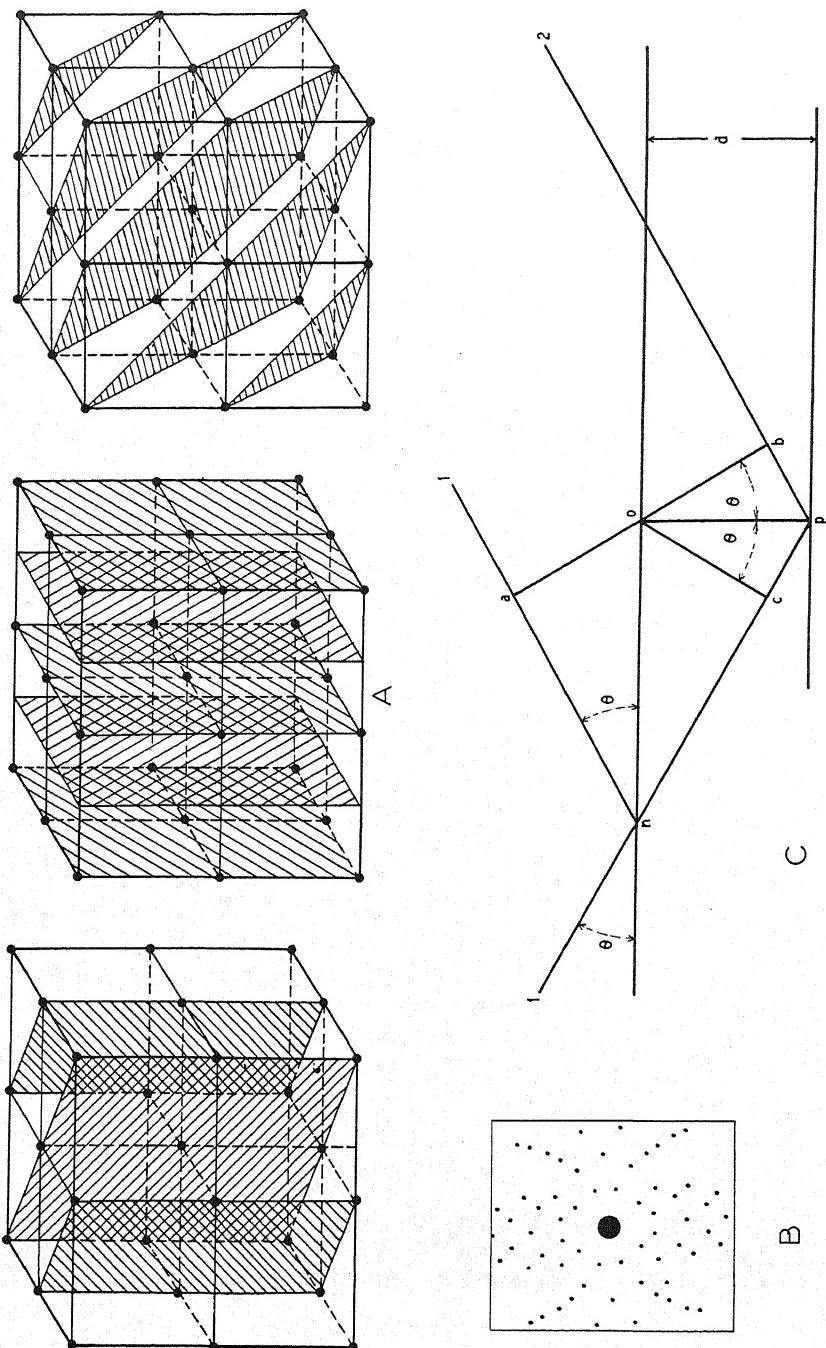
FIGURE 1. Crystal structure and X-ray diffraction: (A) typical sets of parallel planes in a cubic lattice; (B) sketch of symmetrical Laue pattern of single crystal of iron (after Clark); (C) derivation of Bragg law: $n\lambda = 2d\sin\theta$.
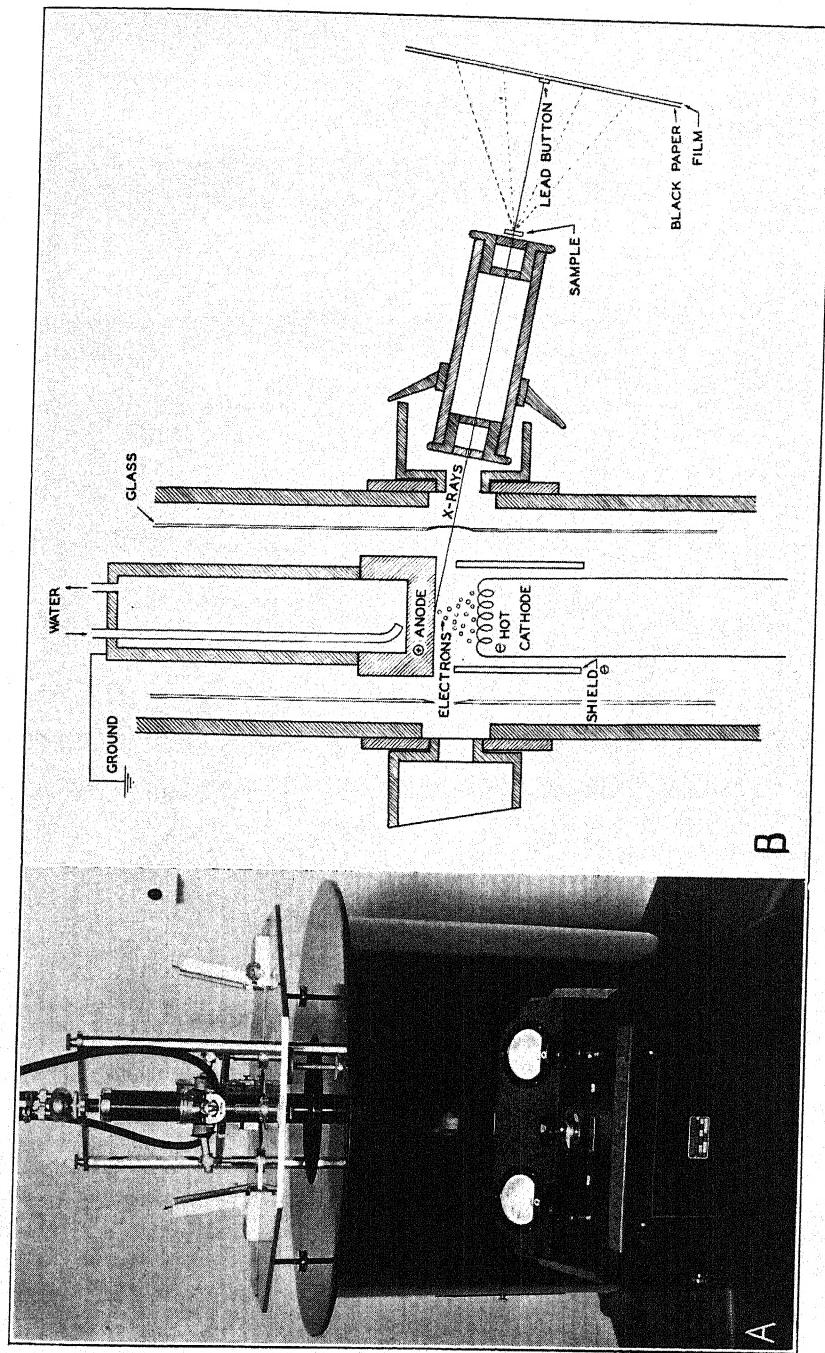
FIGURE 2. Equipment and experimental technique: (A) Photograph of X-ray equipment; (B) cross-section of X-ray tube, slit system, sample and photographic film.

transformer is mounted inside the cylindrical case, and the X-ray tube on top in a vertical position. The slit system and the holder for the X-ray film are also shown in the photograph.

Figure 2 B is a sketch of the X-ray tube. The lighted tungsten filament gives off negatively charged electrons which bombard the positively charged copper anode to produce X-rays. The X-rays emerge from the tube through a thin glass window, and pass through two slits which produce a parallel beam. The parallel beam passes through the sample, and the diffraction pattern is registered on the X-ray film.

The short wave-length X-rays used in making radiographs can not be used for diffraction work. A softer monochromatic beam is necessary. Copper radiation, generated at 28,000 volts, with a characteristic wave-length of 1.54 Å was used for the present work.

In order to illustrate X-ray diffraction analysis, it will be applied to the study of young cotton fibers (*Gossypium hirsutum* L.). To prepare the sample for analysis, the fibers are dissected from the seed, arranged in a parallel bundle and allowed to dry. The bundle is then mounted in front of the X-ray beam in the position shown in Figure 2 B.

By the use of a special micro-technique it is possible to photograph single fibers, but the usual technique employs a bundle of fibers about 1 mm. thick.

After a one-hour exposure, the pattern shown in Figure 3 A is obtained from a bundle of 25-day-old cotton fibers. The black spot in the center is the undiffracted beam. The white area around it is the shadow of the lead cup used to stop the undiffracted beam. Farther out are several rather vague diffraction rings superimposed upon a fogged background.

INTERPRETATION OF DIFFRACTION PATTERN

What information regarding a young cotton fiber can one obtain from these few diffraction rings and a fogged background?

First, the pattern tells us that both amorphous and crystalline materials are present. To illustrate typical amorphous and crystalline patterns, in Figure 3 B is shown the pattern of a simple hydrocarbon, namely paraffin, in the melted or liquid state. The pattern consists of a broad band, similar to that often observed around the moon. If the liquid is allowed to solidify, the solid gives the pattern shown in Figure 3 C containing definite diffraction rings.

Figure 3 D is the pattern of a complex material, namely lignin, in the dry condition. The pattern consists of a fogged disk. The material is amorphous. Figure 3 E is the pattern of the compound indoleacetic acid, extensively used as a growth-promoting substance, which exists as definite crystals. The pattern consists of many diffraction rings.

Thus, from analogy, it may be concluded that the young cotton fiber

contains both complex amorphous and crystalline materials.

Second, there are several crystalline materials present. If the bundle of 25-day fibers is extracted consecutively with water, chloroform, ammonium citrate, and dilute sodium hydroxide, the extracted fibers give the
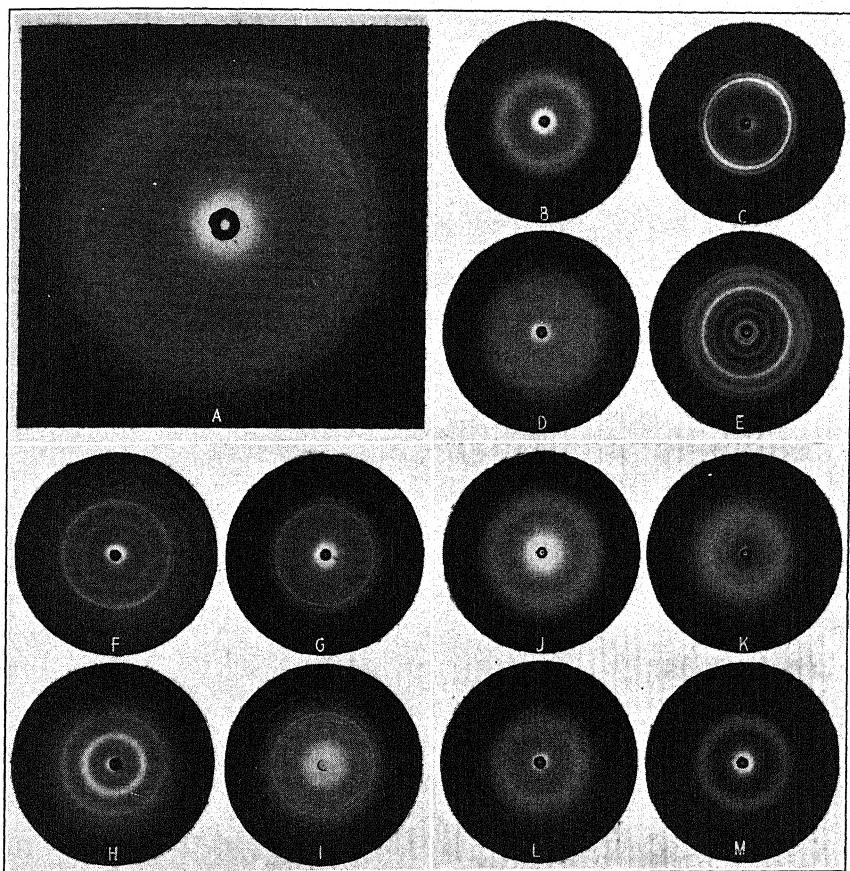
FIGURE 3. Interpretation of diffraction pattern: (A) X-ray pattern of 25-day-old cotton fibers (*Gossypium hirsutum* L.); typical amorphous and crystalline X-ray patterns, (B) liquid wax, (C) solid wax, (D) lignin, (E) indoleacetic acid; representative crystalline cotton fiber constituents, (F) cellulose, (G) waxes, (H) pectic acid, (I) sugars; amorphous fractions extracted from cotton fiber, (J) sodium hydroxide, (K) ether, (L) alcohol, and (M) potassium hydroxide extract of young fibers.

pattern shown in Figure 3 F. From the number of rings, their diameter and relative intensity, this pattern may be identified as that of cellulose. The material recovered from the chloroform extract gives the pattern in Figure 3 G, which is that of a wax. Figure 3 H is the material extracted at 75° C.

with ammonium citrate. It may be identified as pectic acid. If the untreated fibers are allowed to dry slowly at low temperatures, a material crystallizes out which gives the pattern of Figure 3 I. It is probably due to the sugars present in the young cotton fibers.

Third, more than one amorphous material is present. By the use of various solvents it is possible to isolate from the young cotton fibers four fractions which give the amorphous patterns shown in Figure 3 J, K, L, and M. Although it is impossible to identify these constituents from their X-ray diagram, these patterns at least tell us that the materials are amorphous and that they are different.

DETECTION AND ESTIMATION OF PLANT CONSTITUENTS

In addition to identifying plant constituents, X-rays may sometimes be used to estimate approximately the amount present. For example, Figure 4 A contains the diagram of a sample of lignin suspected of containing cellulose. When the lignin was extracted with sodium hypochlorite (Fig. 4 B) the residue gave a definite cellulose diagram (Fig. 4 C). By photographing mixtures containing known amounts of cellulose and lignin, and comparing the patterns with that of the unknown, the conclusion is reached that cellulose is present to the extent of about 2 or 3 per cent.

The unique asset of quantitative X-ray analysis is that the analysis can be made without destroying the sample. Furthermore, it detects types of structure, and not the mere presence of an element. In contrast to spectrographic analysis, X-ray diffraction analysis is far less sensitive. Relatively large amounts must often be present for X-ray detection, especially when dealing with biological materials.

The difficulty often encountered in detecting a substance by its X-ray pattern is illustrated in the case of the young cotton fiber. Figure 4 D, E, F, G, and H shows the X-ray diagram of unextracted young cotton fibers 10, 25, 30, 35 days, and after maturity, respectively. The cellulose diagram does not appear until about the thirtieth day when cellulose is present to the extent of about 35 per cent. The wax diagram, however, which is present at the early stages, does not disappear until it has decreased to a concentration of about 3 or 4 per cent. One might easily erroneously consider these data as proof for the absence of cellulose at the early stages of fiber development.

If the young cotton fibers, however, are treated with a series of reagents to remove all the non-cellulosic constituents, they all give characteristic patterns of cellulose, shown in Figure 4 I.

In this way it has been possible as early as the sixth day to identify cellulose which crystallographically is identical with that of the mature fiber. Cellulose, once it is formed, does not change its crystalline structure with age. Cotton fibers from Egyptian tombs, supplied by Dr. Lansing of
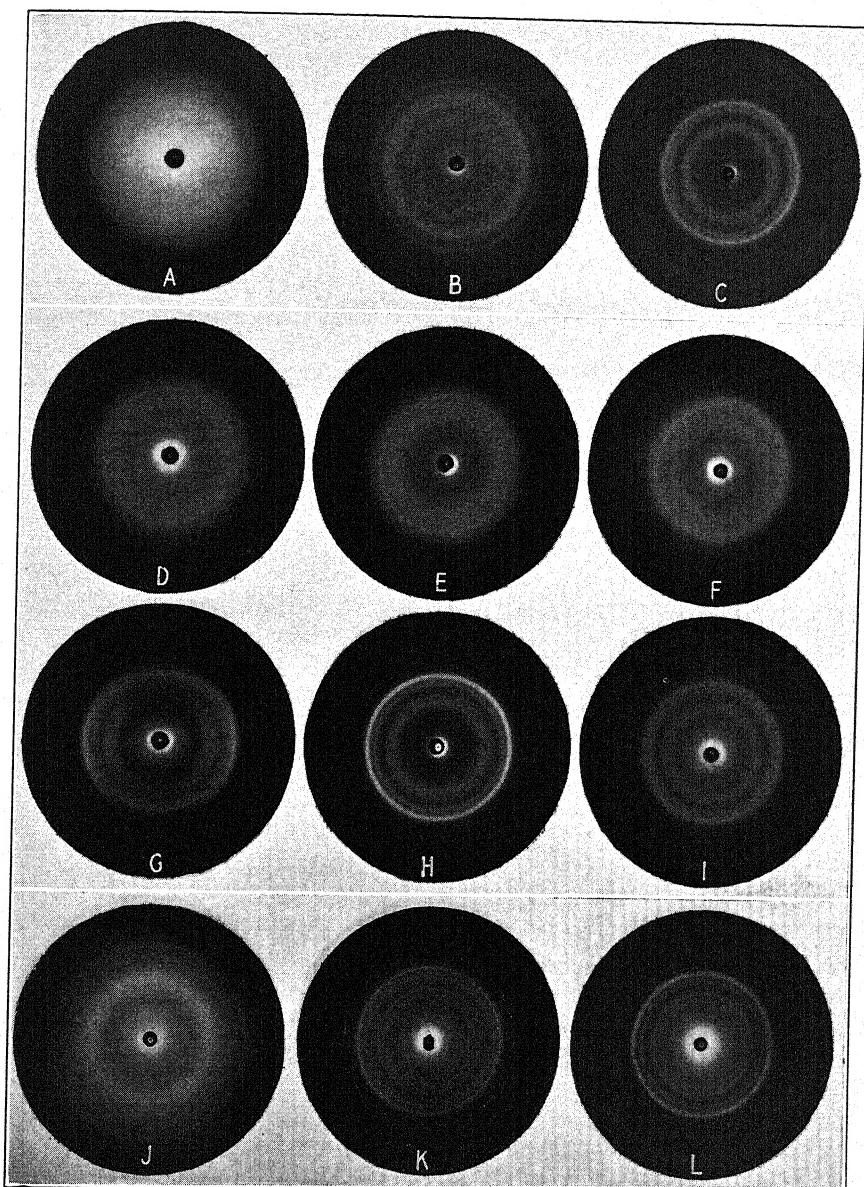
FIGURE 4. Detection and estimation of plant constituents: presence of cellulose in lignin, (A) lignin, (B) cellulose from lignin, (C) cotton cellulose; detection of cellulose in young cotton fibers, (D) 10, (E) 25, (F) 30, (G) 35, (H) 50-day-old fibers, (I) same as (D) after extraction; presence of cellulose in *Valonia* cytoplasm, (J) original cytoplasm, (K) cellulose obtained from cytoplasm by extraction, (L) cellulose from cell wall.

the Metropolitan Museum, have the same crystalline structure after 3000 years.

The small size of the cotton fiber does not readily lend itself to a more detailed study regarding where the crystalline cellulose is first formed. The large cell *Valonia*, however, readily lends itself to a separation of membrane and cytological material. Figure 4 J is the diagram of cytoplasm carefully collected from *Valonia ventricosa* Börgesen. Figure 4 K is the same material after non-cellulosic constituents have been removed. The pattern is the same as that of the cellulose in the mature cell wall shown in Figure 4 L. This indicates that crystalline cellulose is present not only in the cell wall but also in the cytoplasm.

CELLULOSE ORIENTATION IN THE CELL WALL

Another type of information obtainable from the X-ray pattern is whether or not the cellulose has a preferred orientation. To illustrate orientation, Figure 5 A is the diagram of ramie in which the cellulose crystallites, as illustrated diagrammatically above, are oriented parallel to the fiber axis. The diffraction rings, instead of existing as continuous circles, as they do in the pattern of randomly oriented cellulose shown in Figure 5 B, are now concentrated into arcs. The orientation of cellulose in mature cotton lies midway between these two extremes.

An X-ray study of young cotton fibers shows that the preferred orientation associated with mature cotton does not develop until approximately the thirtieth or thirty-fifth day. The diagrams of parallel extracted fibers 25, 30, 35, and 40 days old, are shown in Figure 5 C, D, E, and F, respectively. These diagrams indicate that the cellulose first formed has a more random orientation than that later deposited in the cell wall.

The submicroscopic crystalline structure revealed by X-rays usually runs parallel in the cell wall to microscopically visible configurations such as fibrils. This may be illustrated in the single-celled plant *Valonia*, which was first brought to the attention of X-ray workers by Professor Sponsler. Figure 5 G is a photomicrograph, and Figure 5 H the X-ray diagram of the same section of *Valonia* with the X-ray beam perpendicular to the cell wall. The angle between the X-ray arcs is approximately the same as that between the striations.

In the cotton fiber the striations or fibrils are arranged in a more irregular fashion than in *Valonia*. The width of the X-ray arc in Figure 5 J is a measure of the deviation of the fibrils with reference to the cotton fiber axis as shown in the photomicrograph in Figure 5 I.

The type of orientation in a few miscellaneous fibers is illustrated in Figure 5 K, L, M, N, O, and P. Figure 5 K is flax in which the orientation is parallel to the fiber axis. Figure 5 L is cotton with a rather wide deviation. Figure 5 M is the stem of the cotton plant. Part of the orientation is
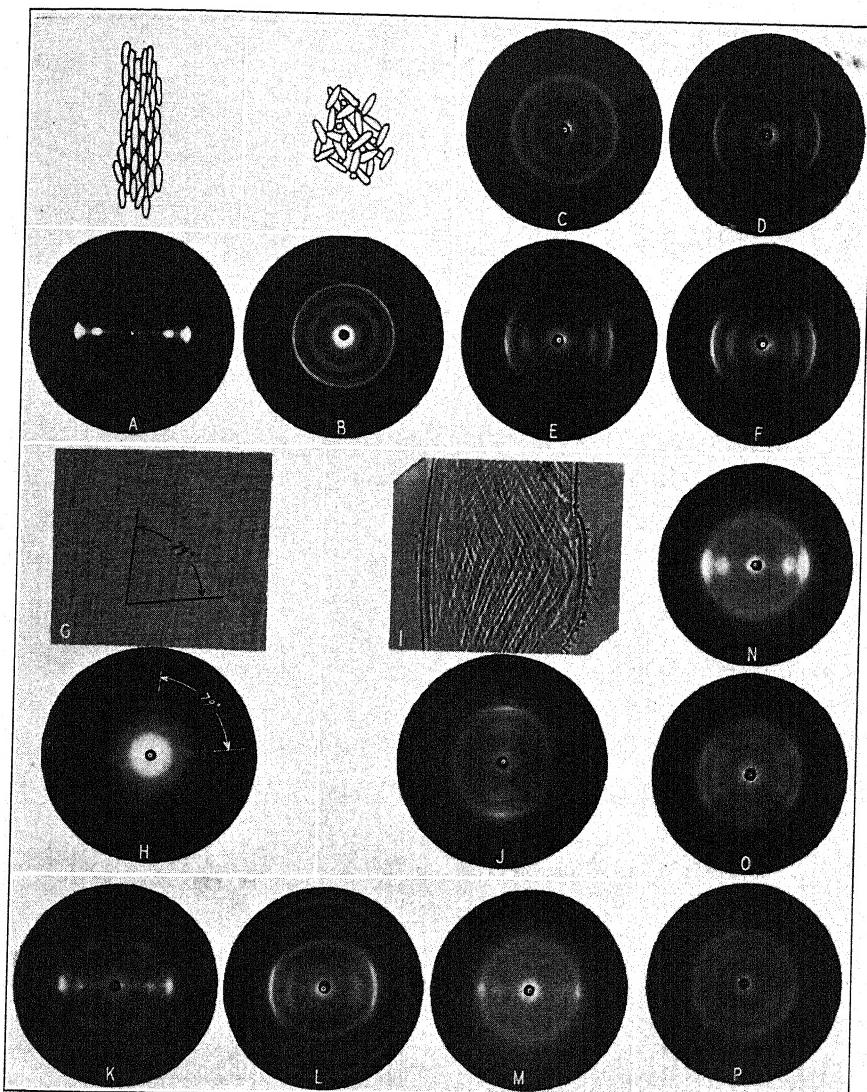
FIGURE 5. Cellulose orientation in the cell wall: X-ray patterns and diagrammatical sketches (after Meyer) illustrating random and preferred orientation, (A) preferred, (B) random; development of cellulose orientation in cotton fibers, (C) 25, (D) 30, (E) 35, (F) 40-day-old fibers; comparison of X-ray and microscopic orientation of *Valonia*, (G) photomicrograph, (H) X-ray pattern, and of cotton, (I) photomicrograph, (J) X-ray pattern; X-ray patterns illustrating orientation in different fibers, (K) flax, (L) cotton, (M) cotton plant stem, (N) summer wood of yellow pine (*Pinus palustris*), (O) compression wood of yellow pine, (P) spring wood of slash pine (*Pinus heterophylla*).

parallel to the fiber axis, but the major portion of cellulose has a random orientation.

The summer wood of normal yellow pine (*Pinus palustris* Mill.) with a fairly parallel orientation is shown in Figure 5 N. Figure 5 O is yellow pine compression wood, which has a constant spiral orientation, giving four arcs similar to *Valonia*. Slash pine (*Pinus heterophylla* Sudw.) spring wood with a random orientation is shown in Figure 5 P.

CRYSTAL SIZE AND STRUCTURE OF CELLULOSE

A further deduction from the X-ray diagram is that the cellulose crystals are very small.

To illustrate the effect of crystal size on the X-ray pattern, Figure 6 A is the Laue pattern of a single crystal of sucrose 1 mm. thick. The pattern is composed of spots. Figure 6 B is the same material ground into smaller crystals approximately 0.1 mm. thick. The spots have decreased in size and increased in number. Figure 6 C is the same material ground into still smaller crystals about 0.001 mm. thick. The spots are now so small and so numerous they form continuous rings. This type of pattern is called a powder or Debye-Scherrer diagram. Figure 6 D is the material after being heated to the melting point and allowed to cool to a glassy amorphous mass. The largest organized unit in this amorphous state is the molecule itself.

Thus, from analogy, since cellulose gives continuous rings (Debye-Scherrer diagram), it may be concluded that the cellulose molecules are organized into larger crystalline building units which are still very small.

Theoretically, it is possible to calculate from the X-ray pattern the size of crystals when their dimensions are within the colloidal range. As the crystal size decreases the diffraction lines get broader. In the pattern of ramie shown in Figure 6 E the diffraction lines are broader in the horizontal than in the vertical direction. On the basis of these data certain investigators have calculated that the cellulose crystallite is a rod-like micelle over 500 Å long and about 50 Å in diameter. These calculations, however, are based upon certain assumptions which, in the light of more recent data, do not seem to be entirely justified.

The exact size and nature of the cellulose crystal, in the writer's opinion, is still an unsolved problem. Whether the cellulose crystal must be considered as a definite micelle (as shown in Figure 6 F), as an area of perfect arrangement of cellulose chains, separated by areas of less organized or amorphous portions (as represented diagrammatically in Figure 6 G), or whether it is a microscopically visible particle (shown in the photomicrograph of Figure 6 H) is yet to be proven.

The type of information to which X-rays make a most useful and unique contribution, is the determination of the unit cell or crystalline
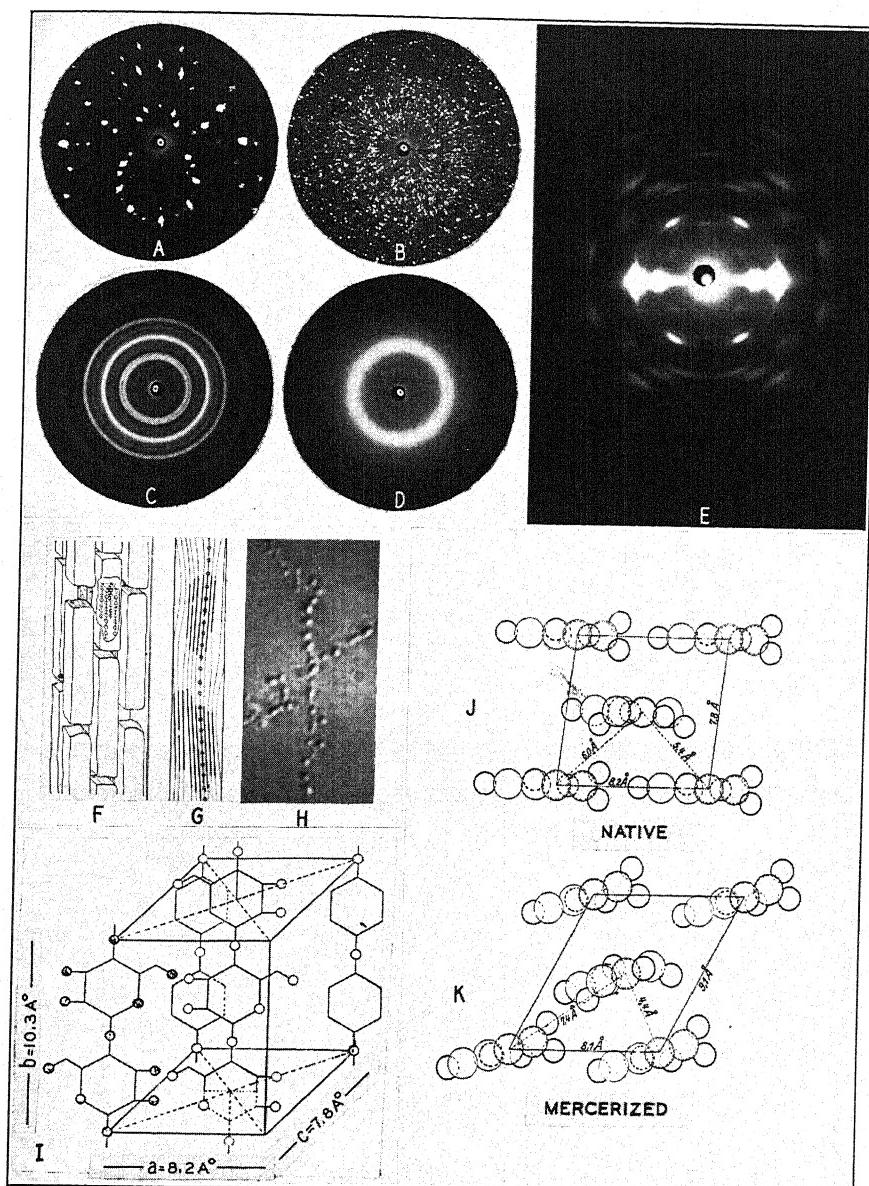
FIGURE 6. Crystal size and structure of cellulose: effect of crystal size on X-ray pattern of sucrose, (A) 1 mm., (B) 0.1 mm., (C) 0.001 mm. thick, (D) same as (C) after melting; (E) X-ray diffraction fiber pattern of ramie; suggested forms of cellulose crystallite, (F) definite micelles (after Meyer), (G) areas of more perfect crystallization (after Sauter), (H) cellulose particles (Farr); crystalline structure of cellulose, (I) side view of unit cell arrangement (after Meyer and Mark), end view of cellulose unit cell (after Andress): (J) native, (K) mercerized form.

structure. Owing to the lack of large single crystals the determination for cellulose is extremely difficult, and some of the details are still unsolved; but from the number, diameter, intensity and position of diffraction spots in a fiber pattern, several independent workers have calculated that the $C_6H_{10}O_5$ units are connected by primary valences to form long chains which have approximately the arrangement with reference to each other shown in Figure 6 I and 6 J.

From this unit cell structure certain basic physical properties of cellulose originate. For example, the arrangement of the molecules in the unit cell explains why cellulose has different refractive indices along different axes, and why it shows extinction in polarized light.

EFFECT OF CHEMICAL REAGENTS ON CELLULOSE STRUCTURE

The vast amount of information that X-ray diffraction analysis has contributed regarding the mechanism of cellulose reactions will be illustrated by the three diagrams shown in Figure 7 A, B, and C. These diagrams, following the course of cellulose acetate formation, show the reaction to be not of the usual homogenous type, but a heterogeneous colloidal surface reaction.

The many special problems to which X-rays have contributed information beyond the range of other techniques are illustrated with the one example, namely swelling.

Figure 7 D is the X-ray diagram of ramie. If the fibers are swollen with water, weak acids or alkalies, the X-ray diagram does not change. If, however, the fiber is swollen with strong alkalies, acids, or certain other reagents, of which the example shown in Figure 7 E is an organic base, then an entirely new diagram is obtained. In the first case the swelling has taken place between the crystallites—intermicellar swelling it is called. In the second case the swelling has taken place between the molecules within the crystallite—it is called intramicellar swelling. Always in the case of intramicellar swelling, the final diagram (Fig. 7 F) obtained after the removal of the swelling reagent is different from the original (Fig. 7 D). The cellulose has assumed a new crystalline structure called the hydrated or mercerized form.

These two polymorphic crystalline forms (native and mercerized), illustrated in Figure 6 J and K by an end view of their unit cell structure, are in a way analogous to the rhombic and monoclinic forms of sulphur. Cloth made of viscose rayon or mercerized cotton has the mercerized type of crystalline structure, that of untreated cotton, the native type.

SCOPE AND LIMITATIONS OF X-RAY ANALYSIS

In this paper attention has been concentrated on one fiber—cotton—and on one constituent of that fiber—cellulose.
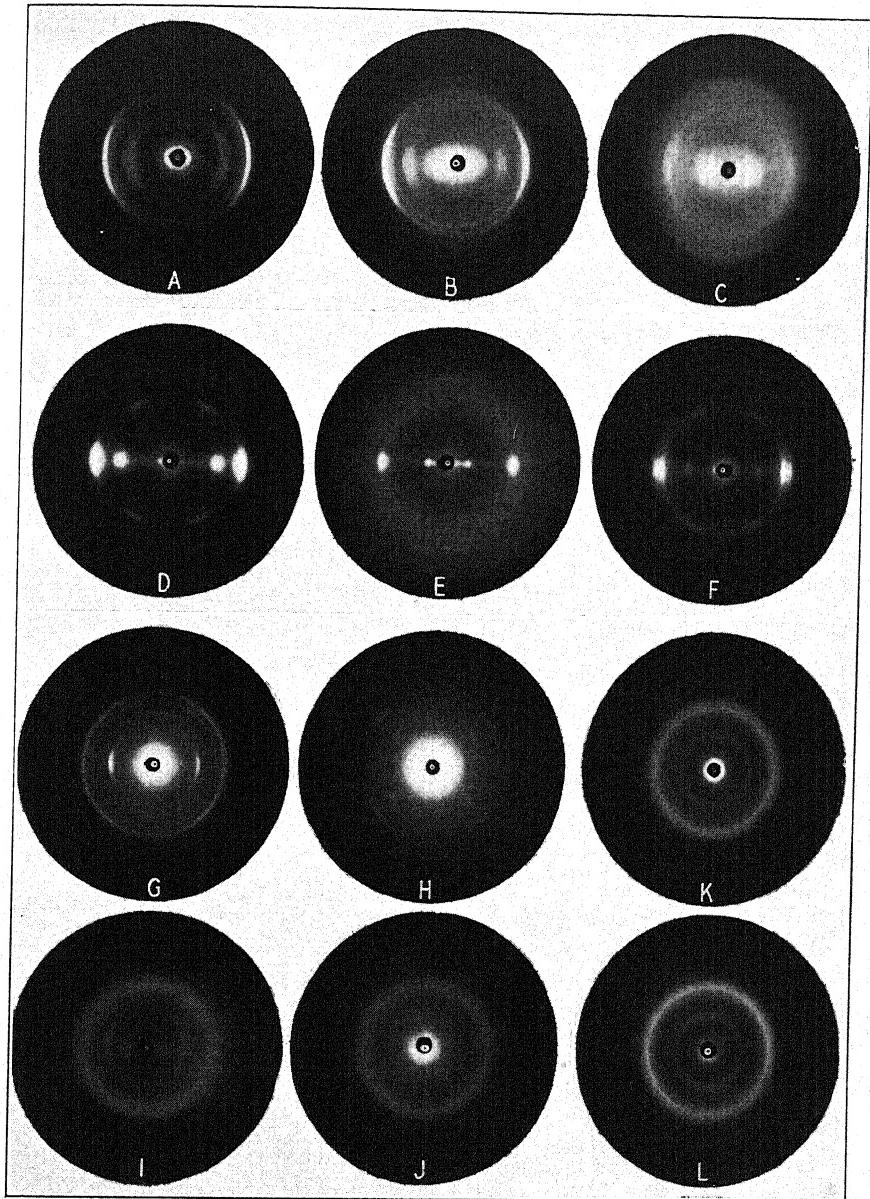
FIGURE 7. Effect of chemical reagents and growth conditions on cellulose structure: formation of cellulose acetate, (A) cotton, (B) cellulose and cellulose acetate, (C) cellulose triacetate; swelling of cellulose, (D) ramie fibers, (E) after swelling with organic base, (F) after removal of swelling reagent; comparison of *Valonia ventricosa* and *Halicystis ovalis*, (G) *Valonia* with X-ray beam parallel to membrane, (H) same as (G) with beam perpendicular, (I) *Halicystis* with X-ray beam parallel, (J) same as (I) with beam perpendicular; mercerized structure of *Halicystis*, (K) X-ray diagram of cellulose from *Halicystis*, (L) mercerized cellulose from cotton.

In discussing the application of X-ray analysis to plant constituents, it is to be remembered that X-ray analysis as a *separate science* is adapted primarily to the study of materials that exist as definite crystals, such as rock salt or steel. For that reason the complicated colloidal materials of biology have been largely avoided by X-ray workers. Furthermore, its application to the study of living materials is greatly limited because of (a) its inability to photograph sections or materials of microscopic dimensions, and (b) the interference of water, which is always present in living materials. In most cases the sample to be examined must be dry. Perhaps the newer science of electron diffraction may help in dealing with surfaces and smaller specimens, but here too the presence of water in living materials is still a problem.

Although the method has limitations, X-ray diffraction analysis has certain definite contributions to make. It is the author's belief that the future hope of X-ray diffraction analysis applied to the study of plant constituents lies in a more close cooperation with other techniques.

CORRELATION OF X-RAY AND MICROSCOPIC RESULTS

The use of X-rays in connection with another technique may be illustrated by referring to cooperative X-ray and microscopic work on *Halicystis*. In Figure 7 G and H are the X-ray diagrams of *Valonia ventricosa* with the X-ray beam parallel (Fig. 7 G) and perpendicular (Fig. 7 H) to the membrane. Corresponding diagrams of *Halicystis ovalis* Kuckuck with the X-ray beam parallel and perpendicular are shown in Figure 7 I and J, respectively. Microscopic work in our laboratories has shown that *Halicystis* differs from *Valonia* in the arrangement of the cellulose and in the nature and amount of non-cellulosic materials present. The visible presence of large amounts of non-cellulosic materials readily accounts for the presence of an additional line in the X-ray diagram of *Halicystis* (Fig. 7 I). The microscopic data also explain why the X-ray diagrams perpendicular to the membranes (Fig. 7 H and J) show a difference in orientation. On the other hand, by the use of X-rays, a selective type of orientation which is beyond the range of detection by microscopic analysis is found common to both *Valonia* and *Halicystis* with the beam parallel to the membrane (Fig. 7 G and I).

In addition, the unusual behavior of the cellulose in *Halicystis* is further elucidated by the fact that the cellulose is in the hydrated or mercerized crystalline form. This difference in crystalline structure is illustrated in Figure 7 K which is the diagram of cellulose isolated from the sample of *Halicystis* shown in Figure 7 I. In Figure 7 L, for comparison, is the diagram of mercerized cellulose from cotton. To the author's knowledge, *Halicystis* is the first known example in which nature has deposited cellulose with a mercerized crystalline form. The structure of *Halicystis*,

however, may be thought of as unusual, simply because many members of the plant kingdom are still unexamined. Perhaps the exploration of new species may produce still other unexpected results.

SUMMARY

With the use of X-ray diffraction analysis the presence of amorphous material and the identity of several crystalline materials are demonstrated in the young cotton fiber. The limitations of qualitative and quantitative X-ray analysis are discussed and illustrated in the detection of cellulose in lignin and at the early stages of cotton fiber growth. The development of orientation in the growing fiber is followed, and the X-ray orientation compared with the microscopic structure. X-ray possibilities in the determination of crystal structure, the study of chemical reactions, and the phenomenon of swelling are briefly pointed out. The limitations and possible correlations of X-ray with microscopic data are illustrated with applications to *Halicystis*.
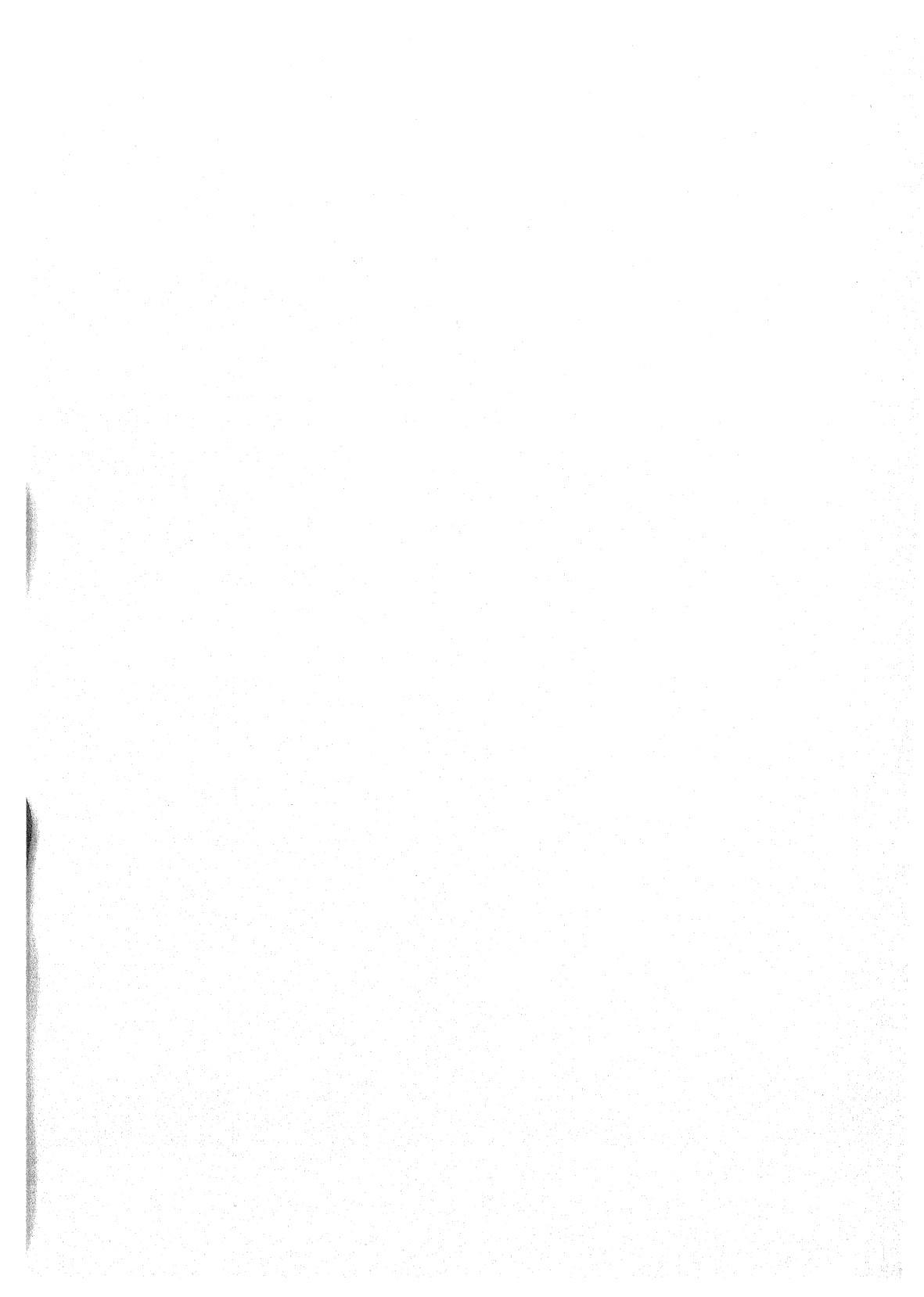
COMBINING TREATMENTS FOR DISINFECTING POTATO TUBERS WITH TREATMENTS FOR BREAKING DORMANCY[1]

F. E. DENNY

Inquiries have been received as to whether potato tubers that had been treated with the usual chemical disinfectants could then be used for dormancy-breaking treatments with ethylene chlorhydrin, sodium thiocyanate, and thiourea.

Experiments undertaken as a result of these requests for information show that the ethylene chlorhydrin vapor treatment (4, p. 284) may be applied successfully to tubers previously disinfected with mercuric chloride, yellow oxide of mercury, or formaldehyde; and further, that tubers treated first with the ethylene chlorhydrin may then be treated with any one of these three disinfectants.

However, when whole tubers previously treated with either of the mercury disinfectants were cut into pieces, they could not then be treated satisfactorily by the ethylene chlorhydrin "dip" method (3, p. 161), since rotting of seed-pieces often occurred; successful results with this method were obtained if formaldehyde was the disinfectant for the whole tubers previous to the application of the "dip" treatment.

Tubers previously disinfected with any of the three disinfectants were suitable for treatments in which the cut tubers were soaked in solution of either sodium thiocyanate (3, p. 158) or thiourea (2).

METHODS

Disinfectants. The treatments with mercuric chloride ($HgCl_2$) and formaldehyde (HCHO) were those recommended by Stuart (5, p. 323, 324), the tubers being soaked 1.5 hours in a solution containing $HgCl_2$ in the proportion of 4 ounces to 30 gallons of water, or for 2 hours in a solution containing formaldehyde (37% HCHO) in the proportion of one pint to 30 gallons of water. The yellow oxide of mercury treatment was that described by Cunningham (1), the dipping solution being in the proportion of one pound of HgO to 15 gallons of water, the suspension being stirred thoroughly before the tubers were dipped into it. After the tubers had been disinfected they were dried thoroughly before being used for the chemical treatments for shortening dormancy.

Dormancy-breaking treatments. For the ethylene chlorhydrin whole-tuber treatments, the tubers were placed in glazed earthenware jars, and the chemical was permitted to evaporate from pieces of cheesecloth spread

loosely upon a watch-glass placed on top of the tubers. The ethylene chlorhydrin used was the usual commercial 40 per cent material. Sealing of jars was accomplished with modeling clay. After the treatments with disinfectant and ethylene chlorhydrin the tubers were allowed to stand in air in paper bags for four days before being cut into pieces and planted. In some tests samples were also stored in this manner for 14 days and the results were similar to those with tubers stored four days.

For the chlorhydrin "dip" treatments the tubers were cut into pieces weighing approximately 25 grams each, which were put in glass Mason jars; they were covered with an aqueous solution containing 50 cc. of 40 per cent ethylene chlorhydrin per liter which was at once poured off and the excess of the solution was dislodged from the potato tissue by shaking the inverted jar; the jar was closed and stored for 24 hours at room temperature.

For the sodium thiocyanate and thiourea treatments the tubers were cut into pieces (as described in the preceding paragraph), and these were soaked for one hour in a solution containing either 10 grams of NaSCN, or 20 grams of NH_2CSNH_2 per liter. At the end of the treatment the solutions were poured off and the treated seed-pieces were planted at once without rinsing.

The treated seed-pieces with their corresponding controls were planted in soil in flats which were stored in tiers at room temperature. At intervals of 4 to 7 days the flats were examined and the seed-pieces showing sprouts above ground were counted and removed. The tables in this paper show the number of days for 90 per cent emergence of sprouts, this criterion being selected as most suitable for evaluating the results, since it serves as a measure not only for rate of emergence, but also for the proportion of seed-pieces which could produce plants.

RESULTS

TREATMENTS OF INTACT TUBERS

The results with the treatment of tubers with ethylene chlorhydrin either before or after disinfection with chemicals are shown in Table I. When the amount of chlorhydrin was 0.4 cc. per 100 g. of tubers (Columns 3 to 6), 28 out of 32 lots showed 90 per cent emergence within 30 days from planting. This seems satisfactory in view of the fact that the control tubers (Columns 11 and 12) required 58 to 120 days to reach this stage. A treatment of 0.2 cc. per 100 g. of tubers (Columns 7 to 10) was successful in 14 out of 16 lots with Bliss Triumph, but showed only 7 successes out of 16 with Irish Cobbler, although these 9 lots, referred to as failures because the 30-day limit was not reached, showed average gains of about 45 days over the corresponding controls.

It is true that in about four-fifths of the comparisons the lots which were not disinfected germinated sooner than those that had been disin-

TABLE I

EFFECT OF TUBER DISINFECTANTS ON THE ETHYLENE CHLORHYDRIN METHOD OF TREATING INTACT TUBERS

Days after harvest when treatments began	Disinfectant used	Time in days for 90 per cent emergence									
		Chlorhydrin treatment 0.4 cc. of 40% per 100 g. of tubers for 5 days				Chlorhydrin treatment 0.2 cc. of 40% per 100 g. of tubers for 5 days				Control, not treated with ethylene chlorhydrin	
		Time of disinfection				Time of disinfection					
		Before		Afterward		Before		Afterward			
		Bliss	Cobbler	Bliss	Cobbler	Bliss	Cobbler	Bliss	Cobbler	Bliss	Cobbler
7	HgCl₂	24	18	19	25	20	23	19	37	98	104
	HgO	31	*	15	13	17	44	24	35	104	120
	HCHO	18	23	24	30	11	17	12	33	82	84
	None	13	18	16	17	14	15	12	27	68	86
14 to 21	HgCl₂	18	24	17	22	14	49	31	38	66	81
	HgO	15	13	24	43	10	47	31	64	60	78
	HCHO	13	24	13	34	14	17	15	56	58	64
	None	13	10	20	29	13	10	26	28	59	62

* 90% emergence not reached because of rotting of seed-pieces.

fected either before or after the chlorhydrin treatment, but if disinfection must be carried out, the data in Table I indicate that the chlorhydrin treatment can be applied successfully, even though the gain in time may not be as much as would be obtained without disinfection.

One reason that the lots disinfected with mercury lagged behind those not disinfected may be found in the fact that the corresponding controls (not treated with chlorhydrin) were delayed in their germination. With the seven-day Bliss lots (Column 11), the delay in mercury-treated lots was about 30 to 36 days, and with the Cobbler lots (Column 12) 18 to 34 days. This retardation due to mercury disinfection was less extensive in the 14 to 21-day lots, and may be an effect produced particularly when the disinfectant is applied to tubers soon after harvest.

There is not sufficient evidence in Table I to show whether it is preferable to disinfect the tubers before treating with ethylene chlorhydrin or afterward. More extensive tests are needed on this point, nor is a preference among the three disinfectants apparent from the results so far obtained, at least in relation to suitability of combination with the ethylene chlorhydrin treatment of intact tubers.

TREATMENTS OF CUT TUBERS AFTER THE INTACT TUBERS
HAD BEEN DISINFECTED

Tubers that had been disinfected previously with each of the three disinfectants were cut into pieces ready for planting and were treated with

ethylene chlorhydrin by the "dip" method, and with sodium thiocyanate and thiourea, with results as shown in Table II.

TABLE II

EFFECT OF DISINFECTANT ON CHEMICAL METHODS OF TREATING THE CUT TUBERS

Variety	Days after harvest before treatment	Disinfectant used with intact tuber	Days for 90% emergence				
			Chemical treatment of cut tubers			Controls in H_2O	
			Chlorhydrin "dip" method 24 hrs.	Soaked one hour			
				in 1% NaSCN	in 2% thiourea	Dip	Soak
Bliss Triumph	15	HgCl₂	*	22	24	100	98
		HgO	*	28	30	100	90
		HCHO	21	19	23	89	92
		None	26	24	23	89	93
	23	HgCl₂	39	25	34	94	85
		HgO	22	27	25	71	69
		HCHO	18	26	29	53	63
		None	18	24	21	46	39
Irish Cobbler	12	HgCl₂	*	25	42	108	101
		HgO	*	28	36	103	113
		HCHO	21	30	43	90	98
		None	21	28	31	80	88
	21	HgCl₂	*	23	36	92	91
		HgO	30	33	35	111	92
		HCHO	17	23	31	72	71
		None	19	21	30	73	71

* 90% emergence not reached because of rotting of seed-pieces.

By the chlorhydrin "dip" method. It is seen (Column 4, Table II) that the chlorhydrin "dip" method was found unsuitable for use with tubers previously disinfected with either HgCl₂ or HgO on account of the rotting of seed-pieces. Disinfecting with either of these mercury compounds, then dipping in H₂O, and storing 24 hours in Mason jars (as is done with the chlorhydrin "dip" treatment) did not produce injury as is shown by the "dip" control (Column 7). But the combined treatments of mercury-disinfection of intact tubers and chlorhydrin dipping of cut tubers caused injury to seed-pieces. The results in Table II indicate that if cut tubers are to be treated subsequently by the ethylene chlorhydrin "dip" method, the previous disinfection of the intact tuber should be done with formaldehyde rather than with mercuric chloride or yellow oxide of mercury.

By soaking in NaSCN or thiourea. Column 5, Table II, shows that tubers previously disinfected with any of the three chemicals were suitable for subsequent treatment with sodium thiocyanate for overcoming dormancy. In only one lot out of 16 were more than 30 days required for 90 per

cent emergence, while the corresponding controls (Column 8) required 39 to 113 days to reach that stage. The results with thiourea (Column 6) were somewhat less favorable than with sodium thiocyanate, but although many of the lots required more than 30 days for 90 per cent emergence, the treated lots showed gains of 18 to 77 days over the lots that were not treated (Column 8).

The thiocyanate treatment not only did not induce rotting of seed-pieces, but in the experiments the results of which are shown in Table III

TABLE III

SODIUM THIOCYANATE TREATMENT OF CUT TUBERS AT INTERVALS AFTER THE DISINFECTION OF INTACT TUBERS

Variety	Treatment of tubers after cutting	Previous whole-tuber disinfectant used	Days for 90% emergence		
			Days after disinfecting before treating with NaSCN		
			0	10	20
Bliss Triumph	Soaked 1 hr. in 1% NaSCN solution	HgCl$_2$	56	38	35
		HgO	56	48	38
		None	54	35	33
	Soaked 1 hr. in H$_2$O	HgCl$_2$	*	107	80
		HgO	*	115	97
		None	*	93	67
Irish Cobbler	Soaked 1 hr. in 1% NaSCN solution	HgCl$_2$	66	53	43
		HgO	52	48	47
		None	67	47	44
	Soaked 1 hr. in H$_2$O	HgCl$_2$	*	*	97
		HgO	*	*	104
		None	*	99	92

* 90% emergence not obtained because of rotting of seed-pieces, final % germination of starred lots first row top to bottom, Column 4 was 48, 60, 58, 84, 66, 68; Column 5 was 76, 80.

the thiocyanate treatment prevented the rotting which occurred in the lots not treated with NaSCN. The tubers for this test were small size second-crop tubers harvested October 22, 1937, and disinfected October 26. Beginning on October 27 and at intervals of 10 and 20 days, samples were removed, cut into pieces, and soaked for one hour either in a one per cent solution of NaSCN or in water. The object was to determine whether the time after disinfecting was a factor in the suitability of the NaSCN treatment of disinfected tubers. The results in Table III (Columns 4 and 5) show that the rot which developed in the control lots when not treated with NaSCN was prevented by the NaSCN treatment. These small and immature tubers were very dormant, and while 90 per cent emergence within 30 days from planting was not obtained in the treated lots, the

gains due to the thiocyanate treatment were 34 to 69 days (Columns 5 and 6).

The time after disinfecting before applying the sodium thiocyanate treatment was not an important factor. If the thiocyanate treatment was not given, however, the time after disinfecting before cutting and planting was important with the immature tubers used in this experiment, rotting of seed-pieces occurring if a delay in planting did not occur after disinfection.

SUMMARY

The germination of recently-harvested potato tubers, after disinfection with mercuric chloride, yellow oxide of mercury, or formaldehyde, was hastened by treating the disinfected intact tubers with vapors of ethylene chlorhydrin (CH_2ClCH_2OH). Also, it was found feasible to treat the tubers first with chlorhydrin and to disinfect them afterward.

Germination was not usually as rapid with tubers that had been disinfected either before or after treatment as with those that were treated with chlorhydrin alone, but the time for 90 per cent emergence of sprouts of disinfected tubers was shortened by 30 to more than 100 days by the chlorhydrin treatment.

A retardation in germination, at least in the period shortly after harvest, was observed when the tubers were disinfected with either of the two mercury compounds, but not treated with chlorhydrin.

Tubers previously disinfected with $HgCl_2$ or HgO were unsuitable for use with the ethylene chlorhydrin "dip" method (by which the tubers are cut into pieces before being treated), since under such conditions much rotting of seed-pieces resulted. Cut tubers responded well to the ethylene chlorhydrin "dip" treatment if the disinfectant previous to cutting was formaldehyde.

Whether the disinfectant previous to cutting was one of the two mercury compounds or formaldehyde, soaking the cut tubers for one hour in solutions of either one per cent sodium thiocyanate or two per cent thiourea hastened germination by 20 to 80 days.

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PROLONGING, THEN BREAKING, THE REST PERIOD OF GLADIOLUS CORMS[1]

F. E. DENNY

In the course of experiments on the use of chemicals in shortening the dormant period of gladiolus corms (2) it was noted that the control corms (not treated with chemicals but merely planted in soil) showed a longer period for germination than would have been expected if the corms had not been planted in soil soon after harvest but had merely been stored in air for a few weeks before being planted in soil. Thus, several varieties showed less than 10 per cent, and four of them zero emergence at six months after planting. This indicated that planting in the soil soon after harvest and storing in moist soil at room temperature furnished the conditions for prolonging the rest period. In the present experiments this point was tested with the same and other varieties and the principle has been verified.

Previous experiments on the effect of storage temperature (1, p. 139) of bulbs in air previous to planting in soil indicated that temperatures somewhat higher than room temperature might be particularly unfavorable for breaking the rest period. A test was made in these experiments to determine whether this delay due to high temperature would occur if the bulbs were stored in soil rather than in air. The results show that with certain varieties an additional delay in germination occurs if the freshly-harvested bulbs are stored in moist soil at $27°$ C.

These experiments indicate that the conditions ordinarily favorable for inducing germination and growth, i.e., moist soil and warm temperature when applied to corms that are not in the rest period, are precisely the conditions for retarding germination when applied to corms that are freshly-harvested and are at the beginning of the rest period.

Corms, the dormancy of which had been prolonged by a suitable choice of storage conditions for approximately seven months, when removed from the soil and treated with vapors of ethylene chlorhydrin, germinated promptly and produced good blooms, while untreated corms of the same lots remained dormant in the soil, in some cases for more than an additional five months.

RESULTS

EXPERIMENTS IN 1937 WITH CORMS HARVESTED IN 1936

The check or control corms from the 1936 harvest showing less than 25 per cent emergence at the end of six months (2, p. 477) were used for

further tests in 1937, and in addition to these, some Halley corms not included in the previous test, but maintained in the dormant condition from the time of planting in October 1936.

On April 12, 1937, corms of Mrs. F. C. Peters, Odin, Halley, and Dr. F. E. Bennett, and on May 11, 1937, corms of Minuet, Mr. W. H. Phipps,
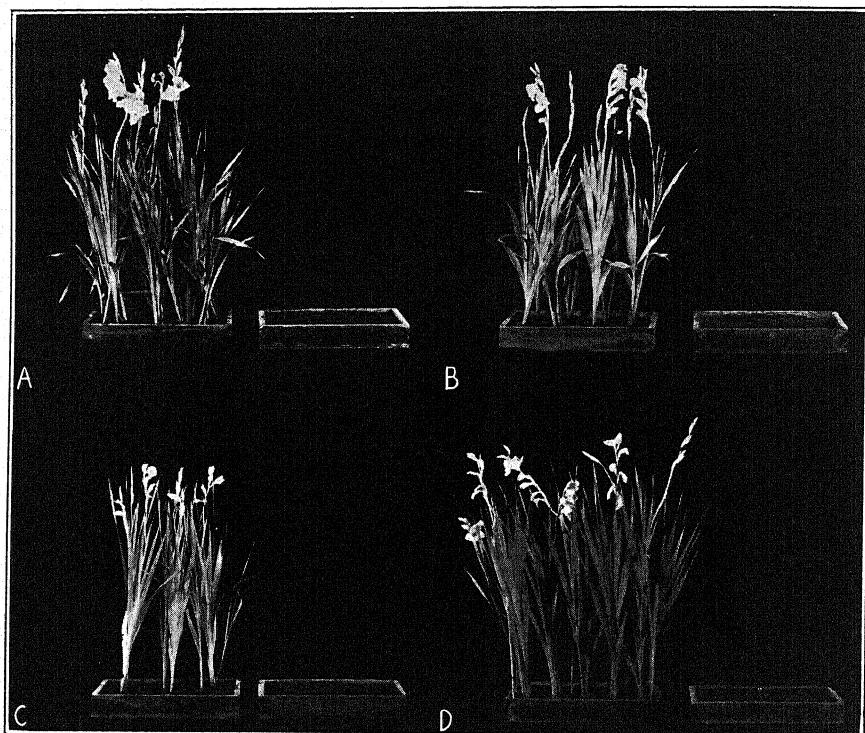
FIGURE 1. Results of treatment in the spring of 1937 of gladiolus corms maintained in the dormant condition from the previous autumn (1936). Corms harvested September 24 to October 13, 1936. Stored in moist soil for 189 to 201 days, i.e., until April 12, 1937 for A, B, and C, and until May 11, 1937, for D. On these dates corms were removed from soil and divided into two lots, one lot (left hand flat for each pair) treated with vapors of ethylene chlorhydrin, other lot (right hand flat for each pair) merely replanted in moist soil. A. Mrs. F. C. Peters, photographed August 26, 1937; B. Dr. F. E. Bennett, photographed August 31, 1937; C. Halley, photographed September 23, 1937; D. Minuet, photographed September 16, 1937.

Senorita, and Mrs. Leon Douglas (192 to 199 days after the original planting) were removed from the soil in the flats and some of them were treated for four days with vapors of ethylene chlorhydrin in closed containers, using 1 cc. of 40 per cent ethylene chlorhydrin per 100 g. of corms. The treated corms were planted in flats, and, for comparison, untreated

corms of the same lots were replanted in flats. The results for the Mrs. F. C. Peters, Dr. F. E. Bennett, Minuet, and Halley are shown in Figure 1. It is seen that the corms of these varieties which had been maintained in a dormant condition for nearly seven months responded at once to treatment with the chemical and produced blooms before untreated corms showed any emergence of sprouts.

The corms of the other varieties, Odin, Mr. W. H. Phipps, Senorita, and Mrs. Leon Douglas, were getting out of the dormant period by this time, and the control lots by September 16, 1937, showed emergence percentages varying from 4 per cent for Odin to 67 per cent for Mrs. Leon Douglas. The days gained by the treatments for the time required to reach the percentage emergences of the control lots varied from 39 days for Mr. W. H. Phipps to 124 days for Odin.

On September 29, 1937, for Mrs. F. C. Peters and Dr. F. E. Bennett, and on October 15, 1937, for Minuet, i.e., approximately one year after harvest, corms not yet germinated were removed from flats and were treated with the chemical. Prompt germination of corms of Mrs. F. C. Peters and Dr. F. E. Bennett was obtained but the subsequent growth was spindly and no blooms were obtained.

The Minuet corms gave only 16 per cent emergence, although underground growth of sprouts with the formation of secondary corms was observed. The results with these corms after long storage indicate that the vitality of the corms had been reduced during such extended storage periods in the soil. Possibly, also, the unfavorable light conditions in the greenhouse in the autumn were factors in the failure to obtain good growth of sprouts and formation of blooms.

EXPERIMENTS IN 1937–1938 WITH CORMS HARVESTED IN 1937
Dormancy Maintained in Moist Soil

The experiments with the 1936 crop of corms indicated that by planting the recently harvested corms in moist soil and storing at room temperature the dormancy of the corms could be maintained for many months with practically no sprout emergence whatsoever. This phase of the problem was repeated with corms of the 1937 crop.

Plantings were made in the interval September 27 to October 14, 1937, about one week after harvest for each variety. The flats were stored in tiers at room temperature, and the soil was kept moist by frequent examinations as to the need of water. On April 15, 1938, six months after planting the record showed no emergence of a sprout for the varieties Purple Glory, Halley, Mrs. F. C. Peters, Dr. F. E. Bennett, Scarlet Princeps, Odin, Senorita, Queen of Bremen, and Salmon Star, and for other varieties the percentage of sprout emergence was as follows: Golden Measure—1; Wilbrink—5; Mr. W. H. Phipps—12; Minuet—56.

Thus, the results are consistent with those of the previous year except in the case of Minuet. With this variety the length of the dormant period in moist soil seems to be correlated with the size of the corm, a special test on this point showed 80 per cent germination within 133 days with corms weighing 25 g. each and no germination with corms weighing 8 g. The corms in the 1936 lots weighed 17 g. each and those in the lots showing 56 per cent germination in the 1937 tests weighed 20 g. each.

Storage in Moist Soil and in Air

While the previous paragraphs show that dormancy may be maintained for many months by storage in moist soil at room temperature, they do not show that the dormancy was greater than would have been obtained by storing the corms in air rather than in soil. Data on this point are shown in Table I. The values in Column 2 are for corms planted in

TABLE I

STORAGE OF GLADIOLUS CORMS IN AIR VS. STORAGE IN MOIST SOIL

Variety	% emergence, 6 months after start of experiment		
	Months stored in air before planting in soil		
	0	2	4
Giant Nymph	13	80	85
Golden Measure	1	35	44
Mrs. F. C. Peters	0	20	45
Scarlet Princeps	0	20	40
Senorita	0	80	90
Wilbrink	5	5	40

moist soil approximately one week after harvest and maintained continuously in the soil; those in Column 3 are for corms stored in air for two months and then planted in soil; and those in Column 4 are for corms stored in air four months before planting. In comparing the values in Columns 2, 3, and 4, allowance must be made for a difference of two months in the time during which the corms were under soil conditions favorable for the growth of non-dormant corms. Table I shows that dormancy is maintained better by storage in soil than by storage in air.

Storage in Soil at Room Temperature and at 27° C.

The effect of temperature of soil during storage on the length of the dormant period is shown in Table II. The temperature effect was most pronounced with Karl Volkert, Flaming Sword, and Minuet, these varieties showing much earlier germinations at room temperature than at 27°.

Table II shows that 27° is a more favorable temperature than room temperature for prolonging the dormant period of corms planted in moist soil.

TABLE II

TEMPERATURE OF SOIL DURING STORAGE AND LENGTH OF DORMANT PERIOD
OF GLADIOLUS CORMS

| Variety | % emergence, 6 months after planting in soil | |
| | Temperature of soil | |
	Room temperature	27° C.
Dr. F. E. Bennett	o	o
Flaming Sword	83	20
Giant Nymph	15	8
Halley	o	o
Karl Volkert	46	6
Minuet	56	22
Odin	o	o
Purple Glory	o	o
Queen of Bremen	o	o
Salmon Star	o	o
Senorita	o	o
White Butterfly*	84	46
Zona*	60	36

* After 4 months.

DISCUSSION

This method of prolonging dormancy does not succeed with certain varieties. In addition to those shown in Table II the corms of the following varieties were not maintained in a dormant condition for a period of six months after harvest: Souvenir, Alice Tiplady, Mrs. Frank Pendleton, and Laughing Water.

It is possible that this method may be of practical use as an inexpensive way of storing corms, especially those intended for a late planting in order to produce a late crop of blooms. Also, if bulbs could be held until midsummer and then treated and planted it may be that favorable light conditions for initiating the growth of flower stalks would be obtained in the early autumn months, the object being to produce a crop of blooms in December or January.

SUMMARY

The corms of several varieties of gladiolus were held in a dormant condition for six months or longer after harvest by storing the *recently-harvested* corms in moist soil either at room temperature, or preferably at 27° C.

Corms whose dormancy had been maintained for seven months in soil at room temperature, when removed from the soil and treated with vapors of ethylene chlorhydrin, germinated promptly and produced blooms before the untreated control corms showed any emergence of sprouts.

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BREAKING THE DORMANCY OF SEEDS
OF CRATAEGUS SPECIES

FLORENCE FLEMION

INTRODUCTION

The present study on seeds of various hawthorn species was undertaken to determine why some species fail to germinate the first spring when planted the previous fall. The winter period out-of-doors in this north temperate zone region is sufficiently long and cold to after-ripen the embryos. Some species such as *Crataegus cordata* (2) germinate the first spring, while others such as *C. crus-galli* germinate only after the second spring (2) with a summer in the interim.

While all *Crataegus* embryos are dormant, the prolonged dormancy present in certain species was found to be caused by the stony carpels which prevent the embryos from after-ripening the first winter out-of-doors. The effect of the seed coat can be removed in the laboratory by treatment with concentrated sulphuric acid combined with a short period at high temperature, or by a period of several months at about 21° C. in a moist medium. An understanding of this phenomenon resulted in a method for the production of a high percentage of seedlings the first spring in all the *Crataegus* species studied.

MATERIALS AND METHODS

When collecting the seeds, trees were chosen which were most nearly like the type species. The work was begun in the fall of 1932 and experiments were repeated the two subsequent years on collections made from the same trees. Seeds of *C. flava* Ait. and *C. coccinea* L. were obtained from seedsmen, *C. oxyacantha* L. was collected at Warsaw, New York, while the others were collected in the vicinity of the Institute at Yonkers, New York. The ripe berries were collected in the fall, and after the pulp was removed with the aid of a Hobart mixer, the seeds were washed well and spread out on blotting paper. When thoroughly dry the seeds were stored at room temperature in containers covered with cheesecloth.

Since many hawthorn seeds are empty, cutting tests (average of two lots of 100 seeds each) were made to determine the percentage of seeds containing embryos. In the case of *C. cordata* Ait. (*C. phaenopyrum* Medic.) where the outer coat is very thin, many of the empty seeds float in water and those that do not can be readily sorted out for the walls have collapsed and appear distorted. However, empty seeds of species having hard stony outer coats neither float nor can they be distinguished from seeds contain-

ing embryos. The number of empty seeds in a given species varies greatly with the different trees in a given locality and also with the same tree in different years. In some cases the number of embryos was as low as 32 per cent. Cutting tests were made on all the various lots and the per cent germination obtained was calculated according to the number of seeds containing good embryos. The germination percentages given in the tables and in the text represent averages of duplicate or triplicate lots of 200 or 300 seeds except when otherwise stated.

When the seeds were treated with concentrated sulphuric acid, a quantity of acid equal to about five times the volume of the seeds was used. The mixture was stirred about every 15 minutes. At the end of the treatment period, the acid was removed, the seeds were thoroughly washed in running tap water, and then kept in a large volume of water for at least an hour to remove any remaining acid. Freshly-harvested seeds were never treated with acid until after several weeks of drying at room temperature because with fresh seeds the acid penetrated and destroyed the embryos due to the higher moisture content of the seeds.

For the plantings made in the greenhouse and out-of-doors a mixture of one-third sand, one-third peat moss, and one-third composted sod soil was used. The seeds were planted in flats ($20'' \times 15'' \times 3''$) which were kept at various controlled temperatures or placed out-of-doors in cold frames, either open, covered with a board cover, or mulched and covered with a board cover. For experiments in the laboratory the above mixture or moist granulated peat moss (purchased from Atkins & Durbrow, Inc., New York City) was used as medium. Seeds when fully after-ripened germinate readily at low temperature or within several weeks after being transferred to a warm greenhouse (about 21° C.). Seedlings in the cold frames appear early in spring.

RESULTS

In the fall of 1932, seeds were planted in flats and the flats subjected to various controlled temperatures or placed out-of-doors while some seeds were mixed in moist peat moss and placed in constant temperature ovens in the laboratory. Within six months, a great range of dormancy was observed, for *C. cordata* germinated readily in flats which had been outside over-winter or in controlled low temperatures and also in the low temperature ovens, while *C. rotundifolia* Moench did not germinate under any of these conditions. Intermediates were found between these two extremes as illustrated in Tables I, II, and III. As the work progressed, periods at high temperature, treatments with concentrated sulphuric acid combined with high temperature, or removal of the carpels were found to overcome the effect of the seed coat. The subsequent period required at low temperature prior to germination was found to be about the same as that required by seeds of *C. cordata*. The various species are described more fully below.

TABLE I

PER CENT GERMINATION OF SEEDS OF VARIOUS CRATAEGUS SPECIES WHEN KEPT
IN MOIST PEAT MOSS AT 5° C.*

Species	Time, months	Per cent germination
Cordata	4.5	92
Coccinea	4.5	86
Mollis	6.0	13
Oxyacantha	6.0	22
Flava	36.0	7
Punctata	36.0	0
Crus-galli	36.0	0
Rotundifolia	36.0	0

* Seeds of the 1932 crop. Experiment started December 11, 1932.

TABLE II

PER CENT GERMINATION OF VARIOUS CRATAEGUS SPECIES WHEN SEEDS PLANTED IN FLATS*
WERE SUBJECTED TO VARIOUS CONTROLLED TEMPERATURES AND
SUBSEQUENTLY TRANSFERRED TO THE GREENHOUSE

Pre-treatment		Per cent germination in greenhouse				
Time at 21° C., months	Time at 5° C., months	Cordata	Flava	Punctata	Crus-galli	Rotundi-folia
0	0	0	0	0	0	0
	3	39	0	0	0	0
	4	74	—	—	—	—
	5	68	0	0	0	0
	7	—	0	0	0	0
	9	—	1	0	1	0
2	5	—	34	26	47	24
4	4.5	—	43	60	73	50
3.5**	5	—	41	51	65	44

* Experiment started December 1, 1932.
** This series had 5 months at 5° C. resulting in no germination, prior to the subsequent periods at 21° and 5° C.

TABLE III

SEEDLING PRODUCTION OF VARIOUS CRATAEGUS SPECIES WHEN SEEDS WERE PLANTED*
IN FLATS AND KEPT IN A MULCHED COLD FRAME

Species	Per cent germination		
	Spring 1933	Spring 1934	Spring 1935
Cordata	59	—	—
Coccinea	47	49	—
Mollis	37	65	—
Flava	4	53	55
Punctata	0	36	51
Crus-galli	0	54	55
Rotundifolia	0	43	49

* Seeds of the 1932 crop planted November 23, 1932.

SEEDS WHICH RESPOND TO SEVERAL MONTHS AT LOW TEMPERATURE

The seeds of *C. cordata* Ait., commonly known as Washington thorn, germinate within four to six months at 5° C. when mixed in a moist medium. As shown in Table I, 92 per cent germination was obtained in four and one-half months at 5° C. Similar results were obtained when the seeds were held at 10° C. or at a fluctuating temperature of 2° to 8° C. When placed at 1° C., a longer period of time was required for germination. Seeds planted in flats and kept at controlled low temperatures also germinated within three to five months (Table II). In the spring, seeds which had been in flats out-of-doors over-winter produced 59 per cent germination in the mulched frame (Table III), 70 per cent germination in the board-covered frame, and 60 per cent in the open frame. In the mulched frame seedlings appear early and often perish or are injured when the mulch is removed. Germination was never obtained from controls kept in the greenhouse throughout the experiment.

Seeds of *C. coccinea* commonly known as Red Haw when mixed in moist peat moss and kept at 5° C. for four and one-half months gave 86 per cent germination (Table I). When seeds were planted in flats and placed in the various cold frames, the germinations obtained the first spring were as follows: 47 per cent in the mulched frame (Table III), 63 per cent in the board-covered frame, 52 per cent in the open frame, and 0 per cent in the warm greenhouse controls.

SEEDS FAVORED BY SOME TREATMENT PRIOR TO AFTER-RIPENING

The 13 per cent germination shown in Table I was obtained with *Crataegus mollis* Scheele seeds kept for six months at 5° C. while seeds placed at 10° C. at the same time produced 50 per cent. In another series, no germination was obtained at 5° C. while seeds placed at 30° C. for several weeks, then transferred to 5° C., produced 42 per cent germination. Seeds when planted out-of-doors in late November of the same year germinated best in the mulched frame with 37 per cent obtained (Table III) the first spring and 65 per cent the second spring. The open frame produced 2 and 4 per cent respectively while the board-covered frame was intermediate yielding 30 per cent the spring of 1933 and 43 per cent the following spring. None germinated in the controls kept during the entire period in a warm greenhouse.

Seeds of *C. arnoldiana* Sarg. when treated for 0 and 4.5 hours with concentrated sulphuric acid, mixed in moist granulated peat moss and kept for six months at a temperature which fluctuated from 2° to 8° C. produced 7 and 20 per cent germination respectively. A duplicate set kept at 28° C. for one month prior to the period at low temperature produced 0 per cent in the controls and 35 per cent in the acid-treated lot.

A very limited number of *C. tomentosa* L. seeds were mixed in moist peat moss and at the end of two years the following germination results were obtained: 26 per cent at 10° C., 7 per cent at 5° C., and 23 per cent from a lot at 5° C. which had been pre-treated for two weeks at 25° C.

Seeds of *C. carrierei* Vauv. were planted in flats. The germination obtained with the lot kept at 5° C. for five months was 28 per cent, while 56 per cent was obtained in the other lot which was treated for three weeks in a 21° C. greenhouse prior to the period at low temperature.

Seeds of *Crataegus* probably *sanguinea* Pall. were treated for 0 and 2 hours with concentrated sulphuric acid, mixed in moist peat moss and held at 25° C. for 0 and 3 weeks prior to the six months' period at 5° C. No germination occurred in the lots not treated with acid whether placed directly at low temperature or pre-treated for three weeks at 25° C. However, the seeds treated with acid attained 5 per cent germination when placed directly at the low temperature and 73 per cent when held for three weeks at 25° C. and then followed by the period at 5° C.

English hawthorn (*Crataegus oxyacantha* L.) seeds as seen in Tables I and IV when kept at 5° C. for six months in moist peat moss produced only 22 per cent germination. However, seeds given various treatments prior to the transfer to 5° C. germinated much better. Seeds of the 1934 crop with and without acid treatment were mixed in moist peat moss. The results are shown in Table IV. The seeds treated for three hours with acid

TABLE IV

EFFECT OF SOAKING SEEDS IN CONCENTRATED SULPHURIC ACID AND OF HIGH TEMPERATURE ON THE GERMINATION OF SEEDS OF CRATAEGUS OXYACANTHA

Duration of acid treatments, hours*	Per cent germination after 6 months at 5° C. subsequent to various weeks at 25° C.			
	0	3	7	12
0	22	26	61	79
2	56	52	68	—
3	59	81	—	—

* November 12, 1934.

were divided into two lots—one, placed immediately at 5° C., yielded 59 per cent germination within six months, while the other lot kept at 25° C. for three weeks, then transferred to 5° C., yielded 81 per cent within six months at this low temperature. The per cent germination obtained when seeds were treated for only two hours with acid is also shown in Table IV. Seeds not treated with acid and kept at 25° C. for three, seven, and twelve weeks produced 26, 61, and 79 per cent germination respectively when subsequently held at 5° C. for six months. Seeds from the same lot were treated on November 12, 1934, for 0, 2, 3, and 4 hours with acid (Table

V). From each lot some were immediately planted and placed in the various cold frames, some were planted and kept in a 21° C. (70° F.) greenhouse for three weeks and then transferred to the cold frames, while some were kept dry at 21° C. for three weeks, then planted and placed out-of-doors. The mulched frame was the best condition. The percentages given

TABLE V

EFFECT OF VARIOUS METHODS OF HANDLING SEEDS AFTER ACID TREATMENTS ON GERMINATION OF SEEDS OF VARIOUS CRATAEGUS SPECIES WHEN SUBSEQUENTLY PLANTED IN FLATS AND KEPT OVER-WINTER IN A MULCHED COLD FRAME

Species, 1934 crop	Duration of conc. sulphuric acid treatment, hours	Per cent germination, spring 1935							
		Weeks at high temperature following acid treatments					Controls		
		Before planting			After planting		Acid treatments only*		
		25° C.	Room temperature (21° C.)		21° C. greenhouse		Treated at start of test	Treated after 3 wks.	Treated after 6 wks.
		3 wks. in peat	3 wks. dry	6 wks. dry	3 wks. in soil	6 wks. in soil			
Oxyacantha	0	—	12	—	41	—	33	—	—
	2	—	32	—	71	—	32	—	—
	3	—	23	—	64	—	29	—	—
	4	—	28	—	65	—	41	—	—
Crus-galli	0	39	1	—	15	—	0	—	—
	2	46	6	—	65	—	5	14	—
	3	48	4	—	68	—	6	9	—
Rotundifolia	0	20	0	0	2	8	0	0	0
	2.5	38	10	8	63	85	10	9	1
	3.5	53	9	9	70	91	18	5	3

* Immediately after acid treatment the seeds were planted and placed in cold frame.

in Table V were obtained in the spring of 1935. The series which had been kept damp in the greenhouse for three weeks after planting prior to the transfer out-of-doors gave the best germination. The series which had been stored dry at room temperature for three weeks gave poorer results than the series which was planted immediately, probably because the latter had received an additional three weeks of after-ripening temperature. Even though the series kept in the greenhouse lost three weeks of low temperature, the period at 21° C. in damp soil was very beneficial in that the highest per cent germination was obtained by this treatment.

While the seeds described above germinate at 5° C. the per cent germination can be greatly increased by various pre-treatments. In other species, such as *C. crus-galli* or *C. rotundifolia*, germination rarely occurs unless given one or both of the above described pre-treatments.

SEEDS REQUIRING TREATMENT PRIOR TO AFTER-RIPENING

Intact seeds of the 1932 crop of *C. flava*, *C. punctata* Jacq., *C. crus-galli* L., and *C. rotundifolia* mixed in moist peat moss were kept for three years at 1°, 5° (Table I), and 10° C., and at a fluctuating temperature of 2° to 8° C. Except for 7 per cent in the case of *C. flava*, no germination occurred. At the same time plantings in flats were made, some of the flats were kept at a controlled temperature (Table II) while others were placed out-of-doors in the various cold frames (Table III). When the seeds in flats were
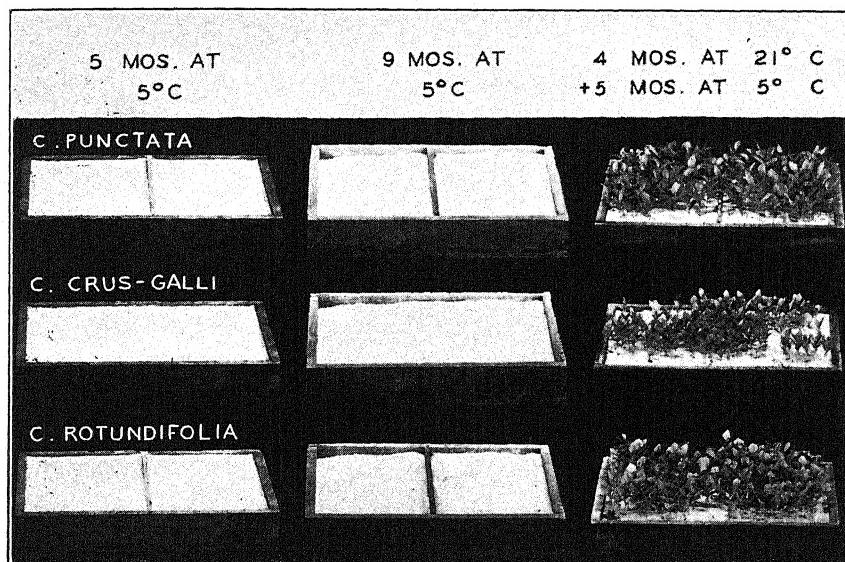
FIGURE 1. Seedling production of various hawthorn species. Lots of 500 seeds each were planted on Nov. 26, 1932, in flats and kept for various periods at 5° C. with and without a previous four months at 21° C. Photographed six weeks after being transferred to a warm greenhouse.

kept at 5° C. for 0, 3, 5, 7, and 9 months, practically none germinated when transferred to the greenhouse. However, when kept at 21° C. for several months prior to the period at 5° C., excellent germination percentages were obtained. The lot in this series which had been at 5° C. for five months produced no seedlings even after three and one-half months at 21° C., but when followed by another period at low temperature, seedlings appeared within several weeks when subsequently transferred to a warm greenhouse (Table II). In a similar experiment the efficacy of a pre-treatment at 21° C. is illustrated in Figure 1.

Seeds planted in flats and placed out-of-doors responded in a similar manner. The results for three successive springs are shown in Table III.

The results were negative the first spring. In these experiments germination occurred only after a long period at high temperature (summer of 1933) which was followed by the cold winter months. No appreciable increase in per cent germination (except for *C. punctata*) was obtained by keeping the flats until the third spring. Conditions in the mulched frame were best as evidenced by the germination results shown in Figure 2. An
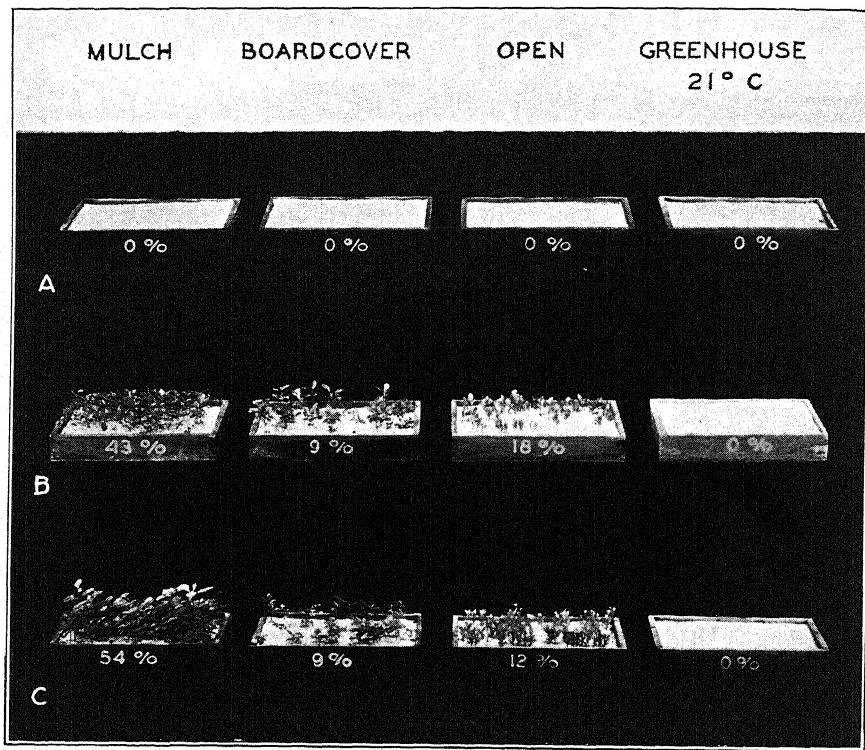
FIGURE 2. Per cent germination of various hawthorn seeds when planted in the fall of 1932 and placed in various cold frames. A and B. *C. rotundifolia* photographed June, 1933 and July, 1934 respectively. C. *C. flava* photographed July, 1934.

even temperature is maintained throughout the winter under the mulch while in the board-covered or open frames the temperature fluctuates considerably. No germination occurred in the controls kept throughout the experiment in a warm greenhouse.

Seeds of *C. crus-galli* and *C. rotundifolia* were treated with acid for various periods of time. Some of the seeds were either stored dry for three and six weeks at 21° C. or kept in moist peat moss for three weeks before planting. The others were planted immediately and kept moist for 0, 3, and

6 weeks at 21° C. before being transferred to the mulched cold frame. The results obtained the following spring are shown in Table V. The acid-treated and control seeds, when placed in the cold frame immediately after planting, germinated very poorly whether the plantings were made following the acid treatments or after three and six weeks of dry storage. The acid treatment did, however, greatly increase the per cent germination when the seeds were subjected to a moist period at high temperature either before or after the plantings (see columns 3, 6, and 7 in Table V). At the time these flats were transferred to the cold frame seeds were treated with acid, planted and placed at once out-of-doors. The negative results given in the last three columns of Table V show clearly the inadequacy of an acid treatment when not combined with a short period in a moist medium at a high temperature.

Some of the *C. rotundifolia* seeds treated on November 24, 1934, for 0, 2.5, and 3.5 hours with acid were sent to Dr. George S. Avery, Jr. of the Connecticut Arboretum at Connecticut College and were planted at New London, Connecticut, on November 28 in flats, and placed in mulched, board-covered, and open frames, and in a warm greenhouse. Another similar lot was sent to him after being kept in our laboratory for three weeks at 25° C. in moist peat moss. The results obtained the following spring were similar to those obtained in Yonkers. The highest per cent germination obtained was from the lot which was held in a moist medium for three weeks at high temperature after being treated for 3.5 hours with acid. No germination was obtained in the non-acid-treated lots. While the percentages obtained at New London were not as high as those at Yonkers (Table V), the results at the two places showed very good agreement.

No germination of intact seeds of *C. rotundifolia* occurred even after three years at constant low temperature (Table I). However, when treated for three hours with acid, then subjected to similar conditions, about 15 per cent germinated within six months (Table VI). When the acid treatment was followed by a treatment of three weeks at a high temperature (column 3, Table VI) in moist peat moss prior to the period at low temperature, over 50 per cent germination was obtained within six months. Seeds planted in flats and kept in the greenhouse (about 21° C.) for 0 and 3 weeks yielded 3 and 19 per cent germination respectively after the period at low temperature. Seeds similarly subjected but pre-treated for three hours with acid showed 17 and 75 per cent germination (Table VI). Intact seeds of *C. flava* were treated with concentrated sulphuric acid for three hours and mixed in a moist medium. One set of duplicates which was placed at 5° C. produced 14 per cent germination while 34 per cent was obtained from the other set kept at 28° C. for one month, then transferred to 5° C. The controls (not treated with acid) placed at 28° C., then at 5° C., showed only 4 per cent germination.

TABLE VI

EFFECT OF ACID TREATMENTS COMBINED WITH SHORT PERIODS AT HIGH TEMPERATURE
ON SUBSEQUENT GERMINATION OF C. ROTUNDIFOLIA SEEDS

Conc. sulphuric acid treatment, hours	Mixed in moist peat moss*		Planted in soil**	
	Per cent germination after 6 months at low temperature		Per cent germ. when subsequently transferred to a warm greenhouse	
	o wks., at 28° C.	3 wks. at 28° C.	6 mos. at 5° C.	3 wks. in 21° C. greenhouse followed by 5 mos. at 5° C.
o	o	2	3	19
1	1	10	—	—
2	7	37	—	—
3	17	47	17	75
4	16	54	—	—
5	15	57	—	—

* 1932 crop treated April 6, 1933.
** 1934 crop treated January 25, 1935.

The effect of stirring the seeds while in the acid was determined by dividing the seeds into three lots. One was stirred continuously, while the other two lots were stirred every 15 and 30 minutes respectively. The lot stirred continuously began to show injury soon after the one-hour period while the two other lots showing only small differences could have been treated longer than three hours. Seeds stirred continuously had to be examined frequently for practically all were destroyed when the injury point was reached shortly after one hour. In the other two lots there was no danger of suddenly ruining all the seeds for signs of injury appeared gradually after about four hours in the acid. For maximum germination results, the seeds should be removed shortly before the injury point is reached and then be followed by a period of several weeks at high temperature in a moist medium prior to the treatment at low temperature. Other chemical treatments were tested but the results were negative.

RÔLE OF THE SEED COATS

Seeds of *C. mollis* with the outer coats removed and inner coats intact gave about 50 per cent germination within six months at 5° C. while the controls with intact coats did not germinate. However, germination of intact seeds of *C. mollis* can be obtained by mixing the seeds in moist peat moss and subjecting the seeds to several weeks at a high temperature prior to the required period at low temperature.

C. flava seeds with hard outer coats removed when kept in moist peat moss for three months yielded 6 per cent germination at 1° C., 63 per cent at 5° C., and 27 per cent at 10° C. Although the embryos require a period

at low temperature, intact seeds never germinate under these conditions. Seeds of *C. punctata*, *C. crus-galli*, and *C. rotundifolia* without any pre-treatment when kept in moist granulated peat moss at various low temperatures rarely germinate while seeds with hard outer coats removed begin to germinate after three months at 5° C. An illustration appears elsewhere (9) showing the behavior of an excised embryo from an intact seed which had remained viable, but had not after-ripened, while in a moist condition for 12 months at low temperature. Even after three years at 5° C. no germinations were obtained with intact seeds (Table I). Subjecting the seeds while in moist peat moss to pre-treatments at high temperature for various periods up to six months increased the per cent germination but the increase was not nearly so great as when the seeds were subjected to high temperature while in the soil. However, the combination of acid treatment and a short period at high temperature was found equally effective whether the seeds were kept moist in soil or in peat moss.

As previously stated, the period over-winter out-of-doors in this north temperate zone is sufficiently long to after-ripen the embryos of *Crataegus*. Seeds adequately pre-treated to remove the effect of the seed coat and then planted in flats and placed out-of-doors in the fall produced results the following spring similar to those in the laboratory. Intact seeds of *C. punctata*, *C. crus-galli*, and *C. rotundifolia* when planted out-of-doors in the fall do not germinate until the second spring. In this way the seeds are subjected to a long period of high temperature during the summer months which is followed by the cold temperature of the second winter.

After three to four months at high temperature in a moist medium the carpels of intact seeds of *C. rotundifolia* and *C. crus-galli* began to split apart at the dehiscent layer. All seeds with split carpels readily germinated when subsequently after-ripened at 5° C. while poor germination was obtained with seeds from the same lot in which the carpels had not split. Moist peat moss was not as effective as soil in bringing about changes in the seed coat and this accounts for the differences in subsequent germination. It was not essential that the two halves of the seed coats be separated for optimum germination. Except for a little carbonized material surrounding the micropyle region, no other visible changes were observed in the dehiscent layer of seeds treated for three hours with acid. The coats of acid-treated seeds were, however, more easily split apart than were intact seeds. It is not known whether the subsequent short period at high temperature further affected the dehiscent layer. The long period at high temperature was eliminated by combining a pre-treatment of acid with a short period at high temperature. Either method resulted in the removal of the effect of the seed coat, thereby permitting after-ripening of the embryos during the subsequent period at low temperature.

RETENTION OF VITALITY

Since the dormancy of *Crataegus cordata* can be easily overcome by a period at low temperature, this species was used in a vitality experiment. Seeds of the 1933 crop were stored dry at room temperature and at three-month intervals duplicate lots of 200 seeds were then after-ripened at 5° C. in a moist medium. Fresh seeds yielded about 90 per cent germination. As seen in Table VII the vitality gradually diminished over a period of

TABLE VII

EFFECT OF DRY STORAGE AT ROOM TEMPERATURE ON VITALITY OF CRATAEGUS
CORDATA SEEDS, 1933 CROP

Storage period, months	Per cent germination at 5° C.	
	After 4 mos.	After 5 mos.
0	54	94
3	84	90
6	90	93
9	89	90
12	77	85
15	74	82
18	75	78
21	42	62
24	47	53
0*	52	88

* Seeds of the 1935 crop.

several years so that seeds stored for two years upon subsequent after-ripening at 5° C. produced only 53 per cent germination. Seeds of *C. crusgalli* and *C. rotundifolia* are no longer viable after being stored dry for three to four years at room temperature. As found for many other seeds, no doubt, the vitality of hawthorn seeds could be considerably prolonged by storing them dry at a temperature around 5° C. (41° F.).

RAPID METHOD OF DETERMINING VIABILITY

Even under optimum conditions the per cent germination of hawthorn seeds is obtainable only after a period of three and one-half to six months at low temperature. A rapid method (9) has been developed whereby the viability of these seeds can be determined within ten days. By observing the behavior of excised embryos which have been placed on moist filter paper in petri dishes at room temperature, the viable embryos can be easily distinguished from the non-viable ones. In this way, the viability can be quickly determined before treating the seeds prior to germination.

DISCUSSION

Delayed germination in seeds is produced by one or more of several causes as described by Crocker (5). He observed (3) that the embryos of

hawthorn seeds were dormant, that is, they would not grow immediately after harvesting, even when the hard stony coats were removed and the seeds with intact testas were subjected to high temperatures or high oxygen pressures. He also found that the coats played a rôle. After removing both coats of *Crataegus mollis* (4) a few of the excised embryos germinated immediately, and others after several weeks. Embryos of *Crataegus* and other rosaceous forms have been induced to grow at temperatures of 22° to 25° C. (8) but the seedlings are dwarfish in appearance, while seedlings from embryos which have been subjected to a sufficient period at 5° C. are normal in their growth habits.

Davis and Rose (6) reported that the cause of delayed germination in *C. mollis* was very largely in the hypocotyls and that 5° to 6° C. was the most favorable temperature in a moist medium for the overcoming of this dormancy. After removing both coats, the excised embryo was dormant in that it required one to two months at this temperature, but when only the hard stony outer coat was removed, the embryo required two and one-half to three months. In their experiments a temperature around 5° C. inhibited the growth of the cotyledons but promoted hypocotyl development. Eckerson (7) with microchemical technique followed the changes occurring in embryos of *C. mollis* during the period at 5° C. She found the storage food to consist mainly of fatty oils and proteins, and as the fats decreased sugar appeared. There was a progressive increase in acidity, in water-holding power, and in catalase and peroxidase activity. This series of metabolic changes occurring prior to germination during the required period at low temperature in a moist medium is spoken of as after-ripening.

Adams (1) working with *C. mollis* seeds obtained only 8 per cent germination following a fall planting while seeds planted in the spring produced almost 50 per cent of seedlings the following spring. The beneficial effect of an initial period at high temperature is again clearly seen. The very low per cent germination obtained by Pammel and King (10) for *C. coccinea* seeds may have been due to a low per cent of embryos in the seeds tested. A great variation was found from year to year in a given species as to the number of seeds containing embryos. They reported (11) no germination for *C. punctata* seeds which had been in the soil out-of-doors for one winter. The failure to get germination in this case may have been due to either of two causes: lack of a warm period in the soil preceding the cold after-ripening period or absence of embryos in the carpels.

The seed germination difficulties encountered among the various *Crataegus* species are readily overcome by the methods suggested above. Under optimum conditions, germination in all species tested is obtainable after three to six months.

SUMMARY

1. All *Crataegus* seeds have dormant embryos which must be subjected to a period at low temperature in a moist medium before germination oc-

curs. Some species have in addition a coat effect which must be eliminated prior to the after-ripening at low temperature.

2. Seedlings of *C. cordata* and *C. coccinea* can be readily obtained after three and one-half to five months at 5° C. or in the spring following a fall planting when kept out-of-doors in a board-covered frame.

3. Although seeds of *C. arnoldiana*, *C. carrierei*, *C. mollis*, *C. sanguinea*, and *C. tomentosa* germinate after a period at low temperature, more seedlings are obtained when the seeds have been treated in a moist medium for several weeks at 21° or 25° C. prior to the low temperature treatment.

4. The per cent germination of *C. oxyacantha* seeds can be greatly increased when the seeds are treated in a moist medium for 12 weeks at 25° C. prior to the subsequent period at 5° C. Another effective pre-treatment is to soak the seeds for three hours in concentrated sulphuric acid and then keep them while in a moist medium for only three weeks at the high temperature.

5. Seeds of *C. flava*, *C. punctata*, *C. crus-galli*, and *C. rotundifolia* planted out-of-doors in the fall do not germinate until the second spring. However, when treatments which overcome the effect of the carpels are applied prior to the fall planting germinations occur the first spring. The temperature maintained throughout the winter in a mulched cold frame adequately after-ripens the seeds of these species.

6. Periods at high temperature in the soil increase the per cent germination of *C. flava*, *C. punctata*, *C. crus-galli*, and *C. rotundifolia*. During the period at high temperature changes occur in the dehiscent layer of the carpel which permit after-ripening to occur during the subsequent period at 5° C. In outside plantings the summer supplies the high temperature. Four months at 21° C. is also excellent. Thus plantings, whether placed under controlled conditions or in cold frames, respond when the period at low temperature is preceded by a period at high temperature. Also, the required period at high temperature can be considerably shortened when the seeds are pre-treated with concentrated sulphuric acid.

7. By combining the acid treatment with a short period at high temperature seedlings can be obtained at any desired time. For maximum germination it is essential that the seeds be in a moist medium during the period at high temperature, for seeds similarly treated but kept dry at this temperature germinate poorly. The subsequent required period in a moist medium at low temperature is obtained either by keeping the seeds at a controlled low temperature or by wintering out-of-doors.

8. Since many seeds require special treatment before the fall planting a duplicate of one experiment with *C. rotundifolia* was sent to New London, Connecticut, and planted there under similar conditions. The efficacy of the pre-treatment was illustrated at both places. The results indicate that such seeds can be pre-treated and shipped to various destinations.

9. By proper treatment seedlings of all *Crataegus* species studied can be obtained on a large scale the first spring after the seeds ripen.

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FORMATION OF β-(2-CHLOROETHYL)-d-GLUCOSIDE BY GLADIOLUS CORMS FROM ABSORBED ETHYLENE CHLOROHYDRIN[1]

Lawrence P. Miller

The results of tests with emulsin have shown that both potato tubers (*Solanum tuberosum* L.) and corms of *Gladiolus* form a chlorine-containing β-glucoside (8) from the chlorohydrin absorbed when these tissues are treated with ethylene chlorohydrin (CH_2ClCH_2OH) in order to break the rest period (3, 4, 5). The acetylation of purified preparations from treated gladiolus corms containing the glucoside formed from the chlorohydrin has resulted in the isolation of a crystalline acetyl derivative which was found to be identical with synthetic β-(2-chloroethyl)-d-glucoside tetraacetate. The latter has recently been synthesized by Jackson (7) and by Coles, Dodds, and Bergeim (2) and has also been prepared in the course of this investigation. These results thus show that the glucoside formed from the ethylene chlorohydrin is β-(2-chloroethyl)-d-glucoside.

EXPERIMENTAL

Isolation of β-(2-chloroethyl)-d-glucoside as the tetraacetate from gladiolus corms. Corms (1914 g.) of the Alice Tiplady variety were exposed in a closed container for 40 hours to a quantity of 40 per cent ethylene chlorohydrin vaporizing from cheesecloth equivalent to 75 cc. of a 0.1 molar solution per 100 g. of tissue. After the treatment period a stream of air was drawn through the containers to give thorough aeration. After a week of aeration the corms were removed from the containers and stored in a paper bag at laboratory temperature. Subsequently they were run through a food chopper, using a fine cutter, and the juice was extracted by squeezing through cheesecloth. The tissue residue was soaked in distilled water and again squeezed through cheesecloth and this extraction with water was repeated once more. The extracts obtained were combined and centrifuged to remove starch. They were then heated to 80° C. and the coagulated material was separated by filtration. An excess of lead acetate was added and after filtering off the lead precipitate the solution was deleaded with H_2S. After removal of the excess H_2S by bubbling nitrogen through the solution, the solution was evaporated under vacuum to a syrupy consistency and was then poured into pure quartz sand and dried in a vacuum desiccator over sulphuric acid.

The residue in sand was then extracted with hot acetone, enough water being added to impart a sticky consistency to the sand-residue mixture.

Four extractions gave a total of 3230 cc. of acetone extract which was found to contain 40.6 millimols of organic chlorine. The acetone extract was evaporated under vacuum and the residue taken up in water. This aqueous solution was then shaken a number of times with ether to remove fatty materials. Some color was also removed by the ether. Extraction with ether is essential for if it is omitted it is very difficult to obtain a crystalline product from the acetylation. After extraction with ether the solution was evaporated under vacuum to a syrupy consistency, and this syrup was heated a number of times with small portions of acetone. Two hundred cc. of acetone extract were thus obtained and analysis showed the extract to contain 27.8 millimols of organic chlorine; calculated as chloroethyl glucoside this was equivalent to 6.05 grams of the glucoside. This solution was evaporated to a low volume under vacuum and evaporated again after the addition of some absolute ethyl alcohol. This was repeated a number of times in order to drive off the last trace of moisture. About 50 cc. of pyridine were then added and the solution further evaporated to remove the alcohol. Sufficient pyridine was then added to make a total volume of about 125 cc. and after the addition of 75 cc. of acetic anhydride the mixture was allowed to stand overnight at room temperature.

The acetylated mixture was poured into five times its volume of ice water with constant stirring. The acetylated product was extracted by shaking a number of times with chloroform. The chloroform solution, also cooled in ice water, was extracted with cold 10 per cent H_2SO_4 to remove the pyridine, then with cold saturated sodium bicarbonate and subsequently several times with distilled water. The chloroform solution, after being shaken with anhydrous calcium chloride was evaporated under vacuum to dryness. On evaporation of the chloroform crystals separated out in the flask. These were recrystallized from absolute ethyl alcohol and 4.49 grams of product melting at 115° C. were obtained. On further evaporation the mother liquor did not give any more crystalline material although analysis showed that considerable organic chlorine was still present. Additional product was obtained from the mother liquor by the following procedure. The mother liquor was evaporated to dryness and dissolved in ethyl ether. On addition of petroleum ether an oily substance separated out. Analysis showed the oily layer to contain only small quantities of organic chlorine. Further addition of petroleum ether resulted in the deposition of crystalline material and in this way 2.29 grams of crude crystals melting at 110° C. were obtained. There was thus obtained a total of 6.78 grams of crude product representing a yield of 59.5 per cent calculating the organic chlorine present in the preparation acetylated as chloroethyl glucoside.

Decolorization with Norite and repeated recrystallization from hot absolute ethyl alcohol gave needle crystals melting at 117.5° to 118.0°

(corrected for stem exposure), optical rotation was $[\alpha]_D^{26} - 21.1°$ in acetone (concn. 3.54 g. in 100 cc.) and $[\alpha]_D^{26} - 13.4°$ in chloroform (concn. 3.86 g. in 100 cc.).

Analyses: Calculated for β-(2-chloroethyl)-d-glucoside tetraacetate, $C_{16}H_{23}O_{10}Cl$; C, 46.78; H, 5.64; Cl, 8.64. Found: C, 46.78, 46.68; H, 5.73, 5.69; Cl, 8.60, 8.46.

Control corms which had not been exposed to chlorohydrin vapor did not contain any organic chlorine.

Preparation of synthetic β-(2-chloroethyl)-d-glucoside tetraacetate. The method used by Jackson (7) was followed. The following equation illustrates the synthesis.

$$
\begin{array}{ll}
\text{BrCH}\text{———}\big| & \text{ClCH}_2\text{CH}_2\text{–O–CH}\text{———}\big| \\
\text{HCOAc} & \text{HCOAc} \\
\text{AcOCH} \quad\quad + \text{HOCH}_2\text{CH}_2\text{Cl} \quad \text{Ag}_2\text{CO}_3 & \text{AcOCH} \\
\text{HCOAc} \quad O \quad\quad\longrightarrow & \text{HCOAc} \quad O \\
\text{HC}\text{———}\big| & \text{HC}\text{———}\big| \\
\text{CH}_2\text{OAc} & \text{CH}_2\text{OAc}
\end{array}
$$
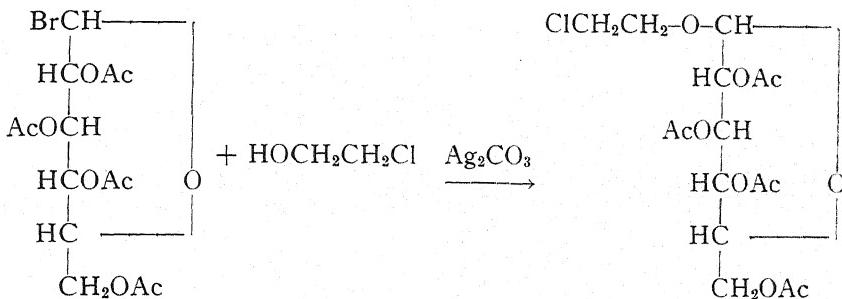
A solution of 41 g. (0.11 mole) acetobromglucose (6) and 134 g. (1.7 moles) of ethylene chlorohydrin (which had been dried over anhydrous sodium sulphate) in 312 cc. of dry benzene was shaken with 41 g. of dry silver carbonate at 8° to 10° C. until the test for bromine was negative. The mixture was then filtered; the filtrate extracted several times with water, dried over anhydrous sodium sulphate and evaporated under vacuum to dryness. The crystalline material in the flask was dissolved in hot absolute alcohol and 26 grams of crystals melting at 117.5° were obtained. This represented a yield of crude product based on the acetobromglucose of about 57 per cent. On two more recrystallizations from absolute ethyl alcohol the crystals melted at 118.5° to 119.0° (corr.). This compares with a melting point of 118.5° to 119.5° (uncorr.) reported by Jackson (7) and a melting point of 114° (corr.) obtained by Coles, Dodds, and Bergeim (2). Optical rotation was found to be $[\alpha]_D^{26} - 21.5°$ in acetone (concn. 4.225 g. in 100 cc.) and $[\alpha]_D^{26} - 13.4°$ in chloroform (concn. 3.525 g. in 100 cc.). Jackson (7) reported a specific rotation of $-13.7°$ in chloroform at 20° and Coles, Dodds, and Bergeim (2) a specific rotation of $-21.25°$ n acetone at 28°.

Analyses: Calculated for $C_{16}H_{23}O_{10}Cl$: C, 46.78; H, 5.64; Cl, 8.64. Found: C, 46.89, 46.92; H, 5.27, 5.31; Cl, 8.44, 8.48.

DISCUSSION

The formation of a glucoside from absorbed ethylene chlorohydrin may be of significance in several respects. (A) The formation of β-(2-chloroethyl)-d-glucoside may be of direct importance in the dormancy-breaking action of ethylene chlorohydrin. (B) Glucoside formation may serve as a means of rendering less toxic the absorbed ethylene chlorohydrin. Thus the effect on dormancy might be due to the dormancy-breaking action of the β-(2-chloroethyl)-d-glucoside formed or breaking of dormancy might result from the effect of the chlorohydrin treatment on the natural glucoside-glucosidase balance. The regulatory action of glucoside systems is stressed by some investigators (1). Preliminary tests on the dormancy-breaking action of chloroethyl glucoside on potato tubers have been unsuccessful because none of the glucoside was taken in by the tubers under the treatment conditions used. More work will have to be done before any conclusions can be reached as to the relation of the formation of chloroethyl glucoside to the dormancy-breaking effect of ethylene chlorohydrin.

Ethylene chlorohydrin is a very reactive substance and it is unlikely that plant tissue could withstand the absorption of appreciable quantities unless some means of detoxication were available. Because natural glucosides often contain aglucons which are very reactive, it has long been thought that glucoside formation may serve to render less active and to stabilize reactive substances arising in the course of plant metabolism (1). The formation of a glucoside from ethylene chlorohydrin shows that plants can also form glucosides from substances which ordinarily do not occur in plants. The animal body has various detoxication mechanisms available which serve to render less toxic or hasten the elimination of introduced poisons. These results with ethylene chlorohydrin show that glucoside formation can be considered a detoxication mechanism in plants.

SUMMARY

The acetylation of purified preparations from gladiolus corms which had been treated with ethylene chlorohydrin gave a crystalline acetyl derivative which was found to be identical with synthetic β-(2-chloroethyl)-d-glucoside tetraacetate. The β-glucoside formed by the tissue from the absorbed ethylene chlorohydrin is thus β-(2-chloroethyl)-d-glucoside.

These results show that plant tissue can form glucosides from introduced aglucons even if such aglucons are substances which do not normally occur in plants. Glucoside formation may thus serve as a detoxication mechanism in plants.

ACKNOWLEDGMENTS

from the gladiolus corms as a means of obtaining a crystalline glucoside, and to Miss H. Jeanne Thompson for performing the micro-analyses.

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LEAF-EPINASTY TESTS WITH VOLATILE PRODUCTS FROM SEEDLINGS[1]

F. E. DENNY

In previous experiments (1, p. 345) seedlings of wheat, corn, and oats failed to give evidence of the production of volatile products inducing epinasty. A continuation of the experiments, however, using other species and arranging the testing procedure to use larger amounts of seeds in smaller containers closed for longer periods has shown that several species during the seedling stage formed volatile products inducing epinasty of potato leaves.

The seedlings of radish and to a less marked extent seedlings of certain other species of the Cruciferae, produced in addition to the epinasty-inducing gas (presumably ethylene) another volatile substance which killed potato plants. This substance is probably mustard oil, formed in the enzymic hydrolysis of glucosides during germination, and it is suggested that this procedure could be included in laboratory exercises in plant physiology as an effective method of demonstrating the presence of mustard oil glucosides in the seeds of this family. Methods of separating the two gases were found by which their effects could be observed separately.

METHODS

Accumulating volatile products. A convenient procedure for accumulating the volatile products from seedlings was as follows: To sterilized two-liter Erlenmeyer flasks stoppered with cotton and containing approximately 250 g. of clean sterilized sand, seeds were sown and covered with another 250 g. of sand, the number of seeds varying with the kind of seed, but representing in most cases the largest number that could be grown successfully in the flask as shown by preliminary tests. These were kept moist with sterile water and stored in a dark room at a warm temperature, usually 22° to 27° C. After the emergence of sprouts, the flask was closed by a two-holed rubber stopper equipped with inlet and outlet tubes. The length of time needed to accumulate volatile products of sufficient concentration to cause epinasty varied with different species, two days sufficing for some and longer periods for others. The period was not extended beyond four days in these tests, this period being used mainly, however, in order to obtain comparisons of various species with radish and other crucifers which gave a response quite different from that of other species included in the tests.

At the end of the accumulation period, the air surrounding the seedlings was transferred by water-displacement to a filter-flask equipped with a one-holed stopper and containing 100 cc. of 20 per cent sodium hydroxide. By shaking the filter-flask for a few seconds the carbon dioxide was removed and the residual air containing the volatile products not absorbed by alkali under these conditions was ready for transference to vessels containing the potato plants used for the epinasty tests.

Control flasks containing sand but no seeds and stored under the same conditions were subjected to the same procedure, but in no case was epinasty obtained. The oxygen supply, the chemical reagents, the vessels used for containing the test-plants, and the rubber stoppers involved in the experiments were tested frequently for absence of epinasty-inducing effect under the conditions of the experiments.

Potato test-plants. A continual supply of young potato (*Solanum tuberosum* L.) plants was maintained by successive plantings in the greenhouse of potato sets in soil in flats. When the sprouts were approximately three inches high with one pair of well-expanded leaf blades, they were cut off at the surface of the soil and put in 5 cc. vials containing water, the sprouts being held in place by a piece of cotton. Usually two of the potato tips were placed in a vacuum-type desiccator of 700 cc. capacity equipped with a lid having an opening for a one-holed rubber stopper. The desiccator was evacuated and an amount of oxygen equal to 20 per cent of the volume of the desiccator was admitted. Then the desiccator was connected with the filter-flask containing the volatile products from the seedlings and allowed to come to air-pressure, the air removed from the filter-flask being replaced by water admitted from a constant-level water supply.

RESULTS

SEEDLINGS CAUSING EPINASTY

In Table I are shown the species which formed volatile products causing epinasty of potato leaves. In this test the flasks were stoppered for four days, but in other tests an accumulation period of only two days was found to be sufficient to give a positive test with such plants as zinnia, bush bean, eggplant, and tomato. The dry weights shown in column 3 of Table I were obtained by removing the seedlings from the flasks at the end of the experiment, rinsing them and drying the tissue in an oven at 105° C.

The epinasty results shown in Table I were obtained by transferring from the two-liter flasks all of the air (except that portion absorbed in 20 per cent NaOH) surrounding the seedlings that had been enclosed in the flask. A number of tests were carried out in which only the gaseous products absorbed by mercuric nitrate were removed from the flasks. The procedure used was a modification of the method of Hansen and Hartman (2, p. 7) and is based on the fact that ethylene is absorbed by mercuric

TABLE I

SEEDLINGS WHICH FORMED VOLATILE PRODUCTS CAUSING EPINASTY OF POTATO LEAVES

Species	Seedlings in 2-liter flask stoppered for 4 days	
	No. of seedlings	Dry wt. at end of test, g.
Tomato, *Lycopersicon esculentum* Mill.	1219	9.11
Eggplant, *Solanum melongena* L. var. *esculentum* Nees.	891	3.21
Lima bean, *Phaseolus limensis* Macf.	10	4.44
Bush bean, *Phaseolus vulgaris* L. var. *humilis* Alef.	39	4.01
Zinnia, *Zinnia elegans* Jacq.	549	3.58
Endive, *Chichorium endivia* L.	3000	3.92
Lettuce,* *Lactuca sativa* L.	1768	1.39
Onion,* *Allium cepa* L.	500	0.97
Muskmelon, *Cucumis melo* L.	335	6.96
Pumpkin, *Cucurbita pepo* L.	25	10.71
Squash, *Cucurbita pepo* L. var. *condensa* Bailey	150	11.10
Turnip, *Brassica rapa* L.	1000	2.12
Brussels sprouts, *Brassica oleracea* L. var. *gemmifera* Zenker.	1000	3.81
Mustard, *Brassica alba* Rabenh.	4100	4.07
Cauliflower, *Brassica oleracea* var. *botrytis* L.	1000	2.61
Broccoli, *Brassica oleracea* var. *botrytis* L.	1000	1.43
Kale, *Brassica oleracea* L. var. *acephala* DC.	3000	7.47
Rutabaga, *Brassica napobrassica* Mill.	828	1.33
Cabbage, *Brassica oleracea* var. *capitata* L.	1000	4.11

* Grown in 700 cc. tube.

nitrate and released from the combination by the addition of hydrochloric acid.

A convenient method of removing the effective constituent and again releasing it was as follows: 100 cc. of a mercuric nitrate-nitric acid solution, made by adding 154 g. of $Hg(NO_3)_2$ and 100 cc. of concentrated HNO_3 to 670 cc. of H_2O, were placed in a filter-flask with a two-holed stopper equipped with a separatory funnel and an inlet tube. The filter-flask was evacuated and filled with the air surrounding the seedlings in the two-liter Erlenmeyer flasks after an accumulation period of four days. The filter-flask was shaken occasionally over a period of five to ten minutes to permit good absorption of the effective constituent in the mercury reagent. Then the filter-flask was evacuated and 40 cc. of concentrated HCl was intro-duced through the funnel. Pure air, from outdoors or from the greenhouse, shown by test not to cause epinasty, was admitted to give atmospheric pressure. Under such conditions ethylene if absorbed by the mercury re-agent is released again as a gas. This gas in the mercury-reagent flask was transferred to a filter-flask containing sodium hydroxide to remove the vapors of HCl and was then transferred to the desiccators containing potato test-plants as described in a previous paragraph.

Seedlings tested and giving positive epinasty responses, when only the volatile fraction absorbed in $Hg(NO_3)_2$ and released again as a volatile

substance by the addition of HCl was used, were: zinnia, onion, radish, turnip, and pepper (*Capsicum frutescens* L.).

A few of the species gave negative or inconclusive tests for epinasty when tested by either of the two procedures described in the preceding paragraphs. The effective volatile constituent, if formed at all, was not produced in sufficient concentration within a single flask to cause epinasty. With some of these a larger number of flasks were used (usually four) and the air from each of the flasks was absorbed successively in the same mercuric nitrate reagent. This served to increase the concentration of the volatile product four-fold or more. When tested in this way the species listed in Table II gave positive responses for epinasty.

TABLE II

SEEDLINGS PRODUCING EPINASTY WHEN THE EFFECTIVE CONSTITUENT WAS ABSORBED FROM SEVERAL FLASKS BY MERCURIC NITRATE SOLUTION

Species	Seedlings in four 2-liter flasks stopped for 4 days	
	No. of seedlings	Dry wt. at end of test, g.
Cowpea, *Vigna sinensis* Endl.	663	70.64
Watermelon, *Citrullus vulgaris* Schrad.	300	24.92
Oat, *Avena sativa* L.	1900	39.04
Millet, *Panicum miliaceum* L.	4400	24.04
Field corn, *Zea mays* L. var. *identata* Bailey	188	51.33
Sweet corn, *Zea mays* L. var. *rugosa* Bonaf.	440	55.53
Wheat,* *Triticum aestivum* L.	2178	42.18

* Six flasks.

Absorption with 87 per cent sulphuric acid. In order to distinguish between ethylene, propylene, and butylene, Hansen and Hartman (2, p. 8) made use of absorption with 87 per cent H_2SO_4, which, according to Tropsch and Mattox (3), absorbs both propylene and butylene but not ethylene. This test was applied to the air surrounding the seedlings of a few of the species used in the present tests. The air that had accumulated around the seedlings during a two- to four-day period was transferred to a filter-flask containing 100 cc. of 87 per cent H_2SO_4 and the absorption was allowed to take place for five to ten minutes, with occasional shaking of the flask. The acid was then carefully decanted from the filter-flask without permitting the escape of any of the air in the flask, the volume of the acid being replaced by admitting pure air shown by test not to cause epinasty. After absorption in 87 per cent H_2SO_4 (and then with NaOH to absorb CO_2) epinasty of potato leaves was obtained with the residual gas from seedlings of zinnia, tomato, lettuce, eggplant, and radish. (The air from the radish seedlings was allowed to stand overnight with 20 per cent NaOH

to decompose the mustard oil before it was absorbed with the 87 per cent H_2SO_4.)

In addition to these tests of 87 per cent H_2SO_4 with seedlings, a few tests were made with the volatile products of other plant parts. Positive epinasty tests were obtained after 87 per cent H_2SO_4 absorption with the air surrounding the following tissues enclosed in flasks: petals of petunia (*Petunia hybrida* Vilm.), leaves of celery (*Apium graveolens* L. var. *dulce* DC), leaves of lettuce (*Lactuca sativa* L.), and leaves of nasturtium (*Tropaeolum majus* L.).

SEEDLINGS PRODUCING VOLATILE PRODUCTS WHICH KILLED POTATO PLANTS

In the routine tests on seedlings in two-liter flasks by the method which gave the results shown in Table I, radish (*Raphanus sativus* L.) responded in a manner quite distinct from the other species. In some tests the usual epinasty of potato leaves was obtained, in others the potato test-plants were killed, in still others neither visible injury nor epinasty resulted. In these early tests the number of seeds used per flask was not rigidly controlled nor was the length of the accumulation period, and later experiments showed that in these factors was the cause of the irregular responses. By planting at least 500 radish seeds in a two-liter flask and allowing a four-day accumulation period in the closed flask, the volatile products in the air surrounding the seedlings consistently caused blasting of the potato test-plants (and also of several other sorts of plant tissue) enclosed in a desiccator. At present it seems likely that the toxic gas formed in the germination of the radish seeds is a volatile mustard oil, allylisothiocyanate ($C_3H_5N:C:S$), set free from the mustard oil glucoside during the germination of the seed and growth of seedling. The air surrounding the seedlings, therefore, presumably contains a mixture of mustard oil and ethylene. When the mustard oil was present in a concentration sufficient to injure the leaf, the leaf could not respond to ethylene by epinastic bending because the toxicity prevented the growth necessary to produce epinasty. When the concentration was properly balanced between the two gases neither epinasty nor a visible injury was obtained, since, apparently, an injury too slight to be observable externally is sufficient to prevent the growth response necessary to cause epinasty. This viewpoint was substantiated by the following observation repeatedly made: When a very toxic gas obtained from radish seedlings was diluted with air by successive steps, a stage was reached at which neither visible injury nor epinasty was obtained, and by still further dilution of this same gas, a stage was reached at which epinasty without injury was obtained. That is, by dilution the mustard oil effect disappeared more rapidly than the ethylene effect. It is

believed that this accounts for the irregular responses obtained in the earlier tests.

Separation of the two gases from each other. The ethylene and the mustard oil were separated from each other as follows: In a filter-flask were placed 100 cc. of the mercuric nitrate solution previously described; by evacuation, air from the Erlenmeyer flask was transferred to this filter-flask, and by shaking the flask the ethylene was absorbed in the mercuric nitrate; the mustard oil was not absorbed by mercuric nitrate and when the air above the mercuric nitrate was transferred to another filter-flask containing 20 per cent NaOH, the CO_2 was removed. This residual gas when transferred to desiccators containing potato test-plants produced the characteristic killing of the potato plant. The injury begins at the tip among the youngest leaves which are at this stage unexpanded, but the blackening spreads to other parts of the plant, which within 24 hours becomes black, liquid exuding from the tissue. The plant finally becomes limp and collapses.

The ethylene was separated from the mustard oil and its effect produced separately by modifying the above procedure at the stage at which the ethylene has been absorbed by the mercuric nitrate solution. In this case the mercuric solution was retained and the air above it (containing the mustard oil) was discarded by evacuation of the filter-flask. The ethylene was then released from the mercuric nitrate combination by adding HCl in the manner described in a previous paragraph. In this way positive tests for epinasty without any sign of toxicity were obtained from samples of radish seedling air which before the removal of the epinasty-inducing constituent caused killing of potato plants.

In quite another way it was found possible to separate the ethylene from the mustard oil. A number of methods were tested for removing the mustard oil without removing the ethylene but the method which finally succeeded was merely to let the gaseous mixture of ethylene and mustard oil stand in contact with 20 per cent sodium hydroxide over an extended period of time, usually overnight. This is the same liquid which was used to remove the carbon dioxide from the gas mixture without removing either the ethylene or the mustard gas. The important factor here is the time of contact. If the gas mixture is shaken with 20 per cent NaOH for only a short period, CO_2 is removed but neither ethylene nor mustard oil is affected perceptibly. But by longer contact with the 20 per cent NaOH the mustard oil is removed, presumably by decomposition, and the ethylene remains in the flask. In this way good epinasty tests were obtained from very toxic samples of the air surrounding radish seedlings.

At first this killing action of the air surrounding seedlings was obtained only with radish. Under the same conditions seedlings of other species of the Cruciferae formed volatile products producing epinasty as shown by

the last eight entries in column 1, Table I, but not causing injury. Since these species contained mustard oil glucosides it appeared likely that between radish and other members of the Cruciferae there was merely a quantitative and not a qualitative difference, and that by arranging to germinate the seedlings in a smaller space the mustard oil concentration might be increased to the point of toxicity. This proved to be the case. Suitable containers for growing these small seedlings were large test tubes approximately 38 cm. × 5 cm. These had a capacity of about 700 cc., but since the sand occupied about 200 cc. the effective volume for gas was about 500 cc., which increased the concentration at least three-fold over that obtainable in two-liter Erlenmeyer flasks. These large test tubes were equipped with two-holed stoppers and the procedure was the same as that previously described for the two-liter flasks.

Using this modification for testing other species of the Cruciferae the results are shown in Table III. Radish appears to be much the most effective of the species tested, but the toxic effect is obtainable from at least certain other species of the Cruciferae.

TABLE III

SEEDLINGS FORMING VOLATILE PRODUCTS WHICH KILLED POTATO PLANTS

Species	Seedlings in 700 cc. tube stoppered for 4 days	
	No. of seedlings	Dry wt. at end of test, g.
Radish, *Raphanus sativus* L.	200	1.41
Mustard, *Brassica alba* Rabenh.	1000	4.11
Cabbage, *Brassica oleracea* var. *capitata* L.	2000	3.55
Brussels sprouts, *Brassica oleracea* L. var. *gemmifera* Zenker.	1000	3.91
Kohlrabi, *Brassica caulorapa* Pasq.	1000	5.99

Tests with mustard oil. Since it appeared likely that the toxic action of the air surrounding radish seedlings was due to mustard oil (allylisothiocyanate) a supply of this chemical was obtained and tests of its effect upon potato test-plants were made. Approximately one drop of the chemical per two-liter flask produced the killing effect upon potato plants in a manner resembling radish-seedling air, both as to the order in which the plant parts were affected and in the final appearance of the plants. Also the vapors of the chemical were rendered non-toxic by standing overnight in contact with 20 per cent NaOH as was the injurious constituent in the volatile products of radish seedlings. In the decomposition of the mustard oil with NaOH no volatile substances causing epinasty were formed.

SUMMARY

Young seedlings of 19 species, growing in flasks which were closed for two to four days to permit the accumulation of gases, formed volatile

products which caused epinasty of potato leaves. The effective constituent was not absorbed by water, 20 per cent sodium hydroxide or 87 per cent sulphuric acid, but was absorbed by a mercuric nitrate-nitric acid reagent, from which combination it was released by the addition of hydrochloric acid. With seven other species the volatile products of seedling growth in a single flask were incapable of inducing epinasty but this result was obtained if the air from several flasks was absorbed in a single sample of the mercury reagent and then released from it.

With various species of the Cruciferae, and especially with radish, the seedlings produced, in addition to the epinasty-inducing volatile product, another gas which killed the potato test-plants. This appeared to be a mustard oil released by hydrolysis of glucosides of the seeds during germination. Methods for separating these two gases and demonstrating their actions separately were found.

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ANATOMICAL CHANGES IN TRADESCANTIA FLUMINENSIS VELL. AFTER TREATMENT WITH GROWTH SUBSTANCES

Robert Bloch

INTRODUCTION

In a previous paper on wound healing in *Tradescantia fluminensis* Vell. (1), anatomical and microchemical changes after wounding were described, and differences in the reactivity of the cells were studied in relation to their position in the various regions of stem and root. It was thought desirable to compare these reactions with those which might be obtained in the intact plant with synthetic growth substances, and also to study the effect of these substances on the wound healing processes.

Histological changes on application of growth substances, such as tissue proliferations and root production, and the reactions of various cell types have been described in a number of recent publications for roots and stems of dicotyledons (7, 9, 10, 13). The present study deals with similar changes in an intact plant of the monocotyledon type with intercalary zones in different stages of maturity. Very little is known as to how growth substances act on processes of cell growth and cell division in comparatively simple structures such as isolated cells and tissue complexes of uniform cells. An attempt to analyze the effects exerted within the complex organization of a whole plant would appear to meet with additional difficulties. There is some indication that correlative and developmental factors play a rôle in the reactions exhibited by the various cell and tissue types somewhat similar to that observed under different conditions as in processes of wound healing and vegetative propagation.

Uyldert (17) applied growth substances to decapitated young internodes of *Tradescantia fluminensis*. Growth and geotropic responses were induced; epinastic and laterally nastic movements of the stem which seem to be linked up with structural characteristics, were difficult to influence. Cooper (4) stimulated root formation in internode fragments of a *Tradescantia* species with β-indolyl-acetic acid. Müller (12) used decapitated shoot fragments of *Tradescantia geniculata*, consisting of a node with parts of the adjoining internodes above and below, or only of immature internode fragments. Roots were stimulated at the nodes, and internodal roots occurred in nodeless fragments. Only a few roots were formed and no anatomical evidence was obtained as to the number of root initials already present before treatment. A paper by Dorfmüller (5) was not seen until the present paper was written. In his experiments also the formation of roots from the internodes served as a measure.

Root formation was also the most conspicuous feature in the present experiments. In order to obtain information as regards the behavior and reactivity of corresponding cells and cell complexes in the various regions of the stem, it was necessary to investigate both early and advanced stages of root formation and to include a brief developmental study of the normal root formation. Growth substances were applied in concentrations high enough to induce characteristic reactions in both young and old internodes and nodes.

MATERIAL AND METHODS

Cuttings consisting of about six internodes were planted in pots in January, 1938, and further propagated in a greenhouse in normal light at a temperature of about 70° F. The plants were not entirely uniform showing a certain variability in the reactions due to differences in the external growth conditions during the experimental period from January to May. Early rooting from the nodes of procumbent stems was prevented by fastening the shoots loosely to sticks so as to allow the upper portion of the stem to grow more or less in its natural position of plagiotropic balance. The epinastic growth and dorsiventral structure of the *Tradescantia* shoot are known to be variable (3, p. 532). They are strong in bright light and disappear in darkness. The shoot, if grown in light, is dorsiventral, bearing slightly asymmetric leaves on opposite sides, and axillary branches which arise in the plane of insertion of the leaves. Roots emerge from the morphological lower side of the nodes. A description of the anatomical and developmental features of the stem is given by Gravis (6), Priestley and Scott (14, 16). Certain changes which take place in older internodes are mentioned by the author (1). The shoot segments were numbered as follows: the first free visible node, the leaf associated with it and the internode below bear number I; toward the base of the shoot the older segments II, III, IV, etc. follow, toward the apex six to seven differentiating segments O_1 to O_6 or O_1 to O_7. The leaves I and O_1 (mostly the 4th and 3rd visible leaves) are fully or nearly fully developed, bearing laminae of about 30 to 40 mm. length and leaf sheaths about 5 mm. long. The internode O_1, which is still hidden within the sheath of leaf I, is generally about 2 to 5 mm. long; longitudinal sections through this region show that above there are still three to four more segments O_2 to O_4 or O_2 to O_5 in the elongating stage, and in them the characteristic meristematic cylinder will be found clearly separated in the peripheral region of the internode between an outer vacuolated cortical and an inner vacuolated perimedullary zone. In the two youngest primordia surrounding the apex, O_5 and O_6 or O_6 and O_7, vein differentiation is still at an early stage both in lamina and axis, and internode extension has not yet commenced; large portions of this region are still meristematic.

Growth substance lanolin mixtures of varying root-inducing power were used (19). Most effective were 0.3 and 0.5 per cent naphthaleneacetic acid, somewhat less effective 0.5 and 1.0 per cent indolebutyric acid, as already reported by Zimmerman and Wilcoxon (20); sufficiently effective 1.0 per cent indoleacetic acid; 0.5 per cent indoleacetic acid did not bring about the changes to be described. Dorfmüller (5) reports that experiments to induce root formation in intact plants of *Tradescantia valida* and *Zebrina pendula* met with negative results. The highest concentration used was 0.5 per cent indoleacetic acid paste. The present experiments show that in Commelinaceae higher concentrations are effective, in particular, of the naphthaleneacetic and indolebutyric compounds.

Plants were treated by applying the paste to the stem above node I, around the leaf sheaths of leaves O_1 and O_2 (Fig. 1 A), or to older parts of the shoot. For each type of experiment an average number of 20 to 25 plants were tested, and in each group the responses were much alike. At definite intervals ranging from 48 hours to 7 weeks the changes in the treated plants were compared with those in the controls which had been left untreated or which had been treated with pure lanolin. The anatomical and microchemical changes were studied in transverse and longitudinal sections made from fresh material and from material fixed in chromacetic acid.

RESPONSES TO TREATMENT

General. In dicotyledons various growth responses occur (18), the first visible phase of which has been described as an epinastic growth reaction of the leaves and stem. In *Tradescantia fluminensis*, a comparable response occurs, though much less vigorous and more local, varying in degree with the age of the leaf, the point of application, and the amount and concentration of substance, 0.01 per cent indolebutyric acid paste still causing a marked reaction. When the paste is applied to the apical region of the stem, the laminae of the leaves O_1, O_2, and O_3 curl downward, and the three or four older leaves below bend downward at the point of insertion to the leaf sheath only. Application of paste to different nodes along the stem makes it clear that there is a gradual decrease in the reaction toward the base of the stem. Immersion of the top of plants into an aqueous solution of indolebutyric acid results in a more rapid response. The plants soon recover after having been taken out of the solution, while the effect of the agent contained in lanolin reaches its maximum effect only after three or more days and may last for weeks. Often the leaves are subsequently dropped in conjunction with secondary changes in the node and internode (Fig. 1 C).

Lanolin treatment at the top of plants (at the level of the apex above node I) modifies the development of the shoot in a characteristic manner.
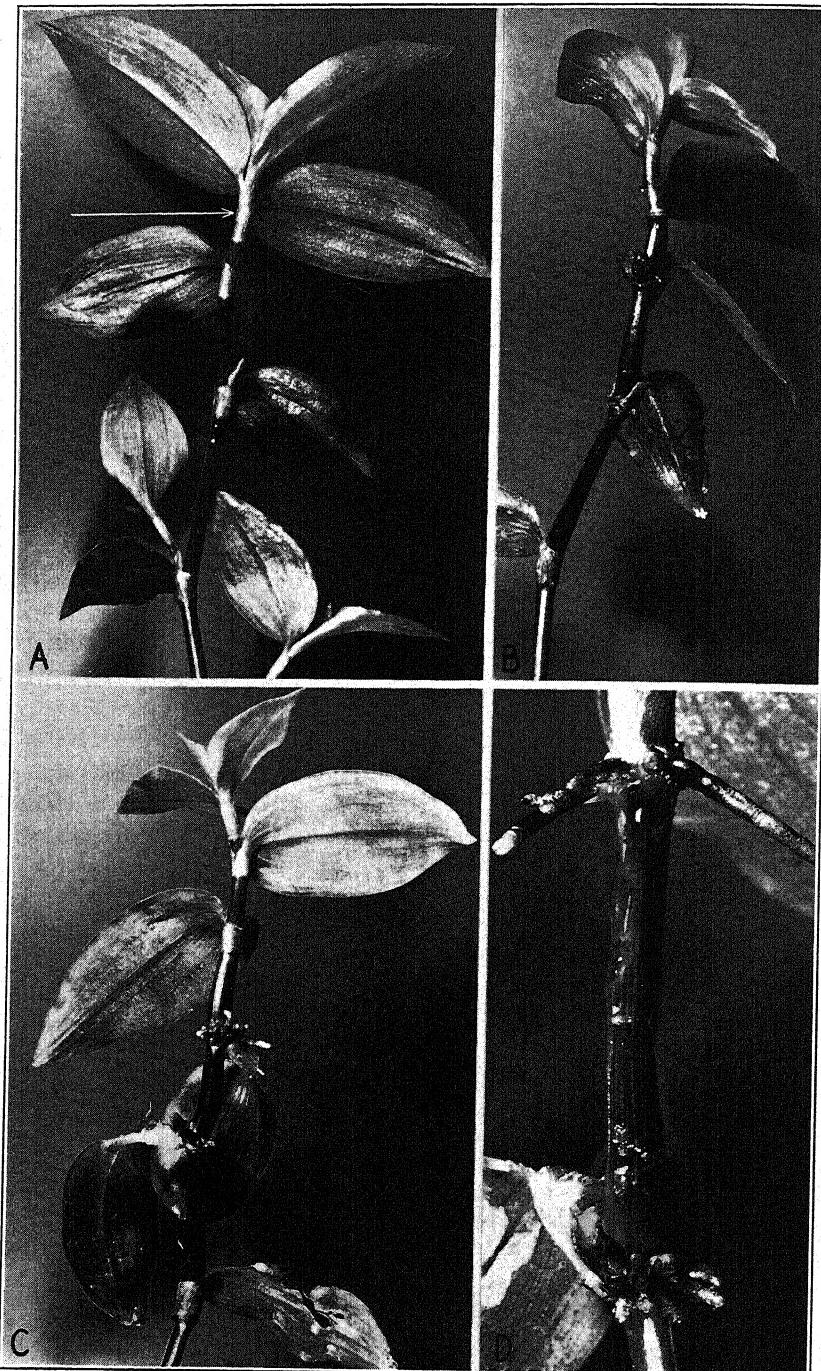
FIGURE 1. (See explanation on opposite page.)

For some time only comparatively short and thick internodes are formed, the basal portions of which simultaneously swell and produce roots, the first external indication of which may be noted after 48 to 72 hours. When the top leaves are previously removed, the newly formed internodes remain thin and short, but root formation is promoted. Soon after the treatment in some of the older nodes at a distance below the point of application already differentiated root initials are vigorously stimulated and new additional roots also begin to appear. When older nodes, e.g., nodes II or III, are treated locally, roots are formed at the point of application and in nodes below. The number of internodes in the elongating region of the shoot, the growth of which is modified, depends on the effectiveness, concentration, and also on the amount of substance externally applied. After treatment with a very effective substance such as naphthaleneacetic acid the successively elongating internodes I, O_1, O_2, O_3 may become affected one after another while the development of the primordia O_4 to O_6 is delayed for several weeks. When these extend subsequently in the normal manner, the laminae generally remain very small. Frequently, however, the plants recover sooner and nearly normal growth is resumed again with internode O_3 (Fig. 1 B, C). Longitudinal sections of the apex taken at definite intervals show that during the retardation period no new primordia are separated. Consequently, as the older primordia complete their development, though modified under the treatment, few or no primordia occur beneath the terminal meristem representing the elongating cycle. This is made more conspicuous by the fact that the internodes whose growth is modified complete their growth in less time than normally required. The length of these internodes is 0.25 to 0.75 per cent of the normal, and the diameter above the node is about 4.5 mm. instead of about 3.5 mm. The growth of axillary buds near the point of application remains suppressed, as is generally known for many species.

EXPLANATION: FIGURE 1

Tradescantia plants showing short and thick internodes and root formation after application of 1 per cent indolebutyric acid paste above node I. (A) Control showing internodes I to IV and axillary branches from nodes III, IV, and V (arrow indicates the point where the paste was applied). (B) Treated on February 15; photographed March 9; fasciated roots arising from original node O_1, roots indicated on node O_2. Sheaths of leaves O_1 and I are burst and a portion of the lamina O_3 is still curled downward; normal growth has been resumed in the upper portion of the shoot. (C) Treated on February 21; photographed March 9, after normal growth had been resumed. Internodes I, O_1 and O_2 are abnormally thick and short and several circles of fasciated and individual roots appear on nodes I and O_1. The originally epinastic leaves I and O_1 are being dropped. (D) Internode III treated on February 16, which caused induction of roots from nodes III, IV, V, and VI. Photographed March 9, showing downward growing roots from node III and 2 to 3 circles of roots arising from node IV. Big root arises near axillary bud (the latter not visible); internode shows swellings both in upper and lower portion.
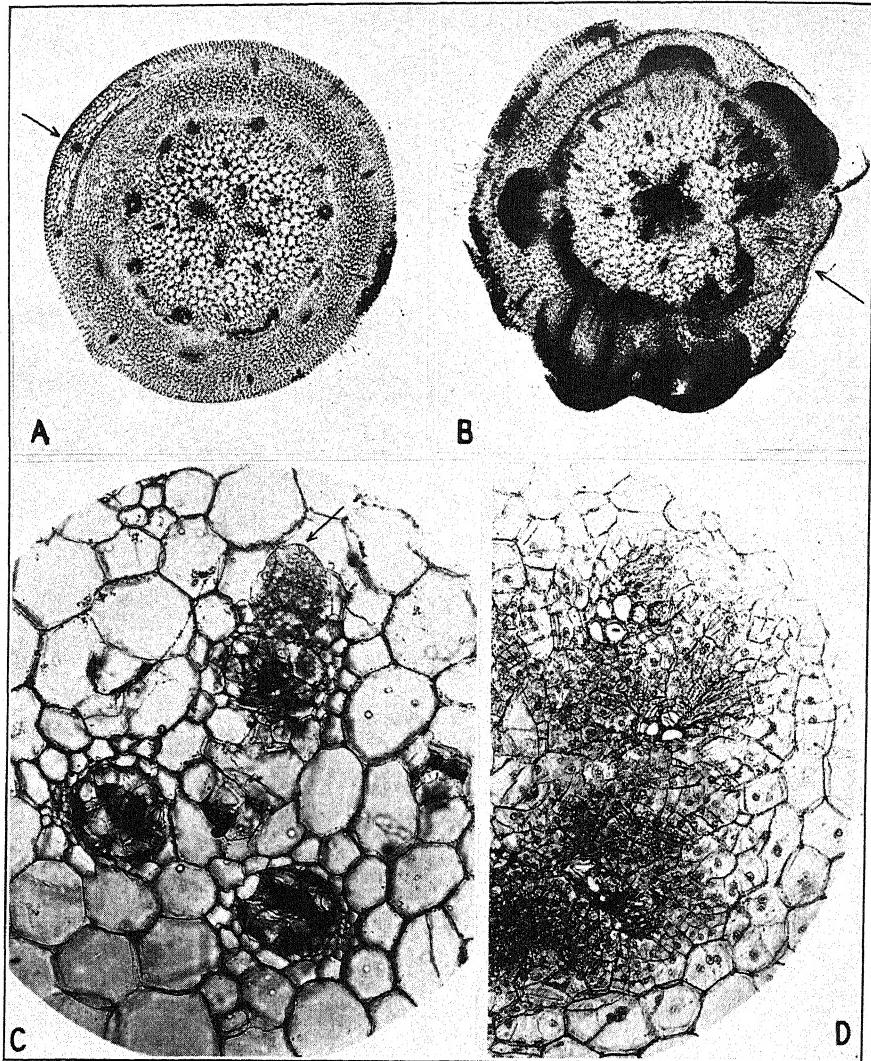
FIGURE 2. Transverse sections of nodes and internodes. (A) Control, normal node IV showing three root primordia and cross connections of tracheids forming on the lower side of the node (arrow points to position of axillary bud). (B) Node I after treatment with 0.3 per cent naphthaleneacetic acid from May 25 to June 10, showing two roots, emerging from lower side of node, three roots from peripheral meristem at the other side, and one root between the medullary bundles above the nodal plate, as in Figure 3 B (arrow points to position of axillary bud). (C) Internode I decapitated about 2 mm. below node I and surface covered with 1 per cent indolebutyric acid from February 18 to April 4, showing tyloses and necrotic deposits in xylem channels of medullary bundles, and root initial outside the phloem of one bundle (see arrow). Section at a slightly lower level than Figure 4 A. (D) Node V treated with 0.3 per cent naphthaleneacetic acid from May 13 to May 24, showing meristematic activity around central bundles above nodal plate.

Histological. Normally the nodal roots make their appearance later than the axillary bud; their formation is linked up with activity of the nodal peripheral meristem and with basipetal differentiation processes in the peripheral region of the internode above (16). Reports in the literature state (15) that roots normally form all around the stem, but in a considerable number of shoots examined only two, sometimes three roots were seen (Fig. 2 A), which in the majority of cases remained the only roots emerging and striking the substratum from the lower side of older nodes of trailing stems. The root initials appear during the later stages of internode extension and are distinguishable in node O_1 or I. They arise some distance above the nodal plate, near the upper end and outside the nodal vascular ring from the peripheral meristematic region which at this level appears somewhat asymmetrically arranged and more active on the ventral side of the node (Fig. 2 A). On this side the formation of a peripheral ring of tracheids begins in the plane of insertion of the root initials also. In old nodes, root initials may form also at the other sides, particularly is this the case, when suitable conditions arise, e.g., at the base of cuttings. Roots may then be formed from basal portions of internodes, as stated by Schubert (15), from cells outside the phloem of the peripheral bundles or the sclerenchyma sheath or surrounding the internal bundles.

As regards the general regenerative capability of the stem tissues of *Tradescantia*, it was shown that when wounded, all living vacuolated cells may return to the meristematic stage and divide and resume growth, particularly the potentially meristematic layers just mentioned (1). The greatest regenerative power is exhibited by the complex of uniform cells composing the vegetative point. Mirskaja (11) has demonstrated for *Tradescantia guianensis* that after injury processes of direct regeneration are initiated in this region.

The present experiments brought out some facts that furnish additional evidence as regards the comparative reactivity of the various meristematic and potentially meristematic zones. Three different regions can be distinquished in the shoot in which cells become differently affected by the treatment, though the transition from one type to another is gradual:

A. In the mature or nearly mature part of the stem (nodes and internodes X to II) local application of paste stimulates vigorously the growth of root initials already present and promotes the formation of many new roots, though there is a gradual decrease in number toward the base of the stem. Such anatomical changes did not occur to a noticeable degree in nodes above the point of application. The roots may emerge in one, two, or three circles above one another (Fig. 1 D, node IV); the average number of roots and root initials occupying one circle was 13 and corresponded with the number of peripheral bundles. The roots of the lowest nodal circle are most readily formed and arise from the peripheral meristem; the roots of

the higher circles are formed from more or less vacuolated cells outside the peripheral bundles. The phloem takes an active part at an early stage of root differentiation; later short tracheids form the connection between the vascular strands of the young root and the xylem of the bundle. Roots are also formed outside the phloem of the internal bundles (cortical, perimedullary, medullary), but do not appear to be able to emerge. Particularly in the nodal region of vascular anastomoses, where there is long continued meristematic activity, root formation reaches a maximum comparable to that proceeding at the same time in the peripheral region. Numerous roots are formed above and below the nodal plate both outside the phloem or around the medullary and perimedullary bundles before these pass into the anastomosing system of the node (Fig. 2 D) and from the meristematic cells abutting on the plate itself.

B. In the rapidly differentiating and extending zone (nodes and internodes I, O_1 to O_3) root formation is very vigorous and distinctly premature when in the still short segment much larger portions of the basal meristem become involved (Figs. 2 B, 3, 4 B). This leads subsequently to conspicuous anatomical differences in the basal peripheral region of the internode. Three to six days after treatment abnormally shaped roots begin to emerge from nodes I and O_1, which also become very rich in vascular elements, and correspondingly later, though very early induced, from the succession of younger internodes and nodes. No special direction of growth prevails, but owing to the asymmetric activity of the peripheral meristem in the node, roots very often were more vigorous at the lower side of the node or in the neighborhood of the axillary bud (Figs. 1 C, D, 4 B). Most varied forms occurred such as a complete circular disk (node O_2); long flat bands or wing-like structures about 10 mm. long and 2 to 4 mm. wide, often enclosing part of the internode like a belt, finally breaking up into individual more or less fasciated roots (nodes O_1, O_2, O_3); thick comb-like plates, arising in the horizontal plane from the lower side of the node and bending upwards or downwards; vertical plates (Fig. 1 C); additional individual roots as described before were formed at the same time or subsequently throughout the length of the internode, though more readily from the differentiating peripheral bundles (Fig. 3 A) and in the central region near the nodal plate (Figs. 2 B, 3 B, C).

C. No appreciable changes appear either in the cells of the meristematic apex or in the two youngest primordia in which, at the time of treatment, the first stages of differentiation into procambial and parenchymatous regions are taking place. Development is distinctly delayed in this region, and microscopical examination of this part three to six weeks later, when normal growth has been resumed, reveals no sign of root-forming activity. Such stimulation is, however, very conspicuous in node O_3, which at the time of treatment occupied the place between the first pair of extending internodes.
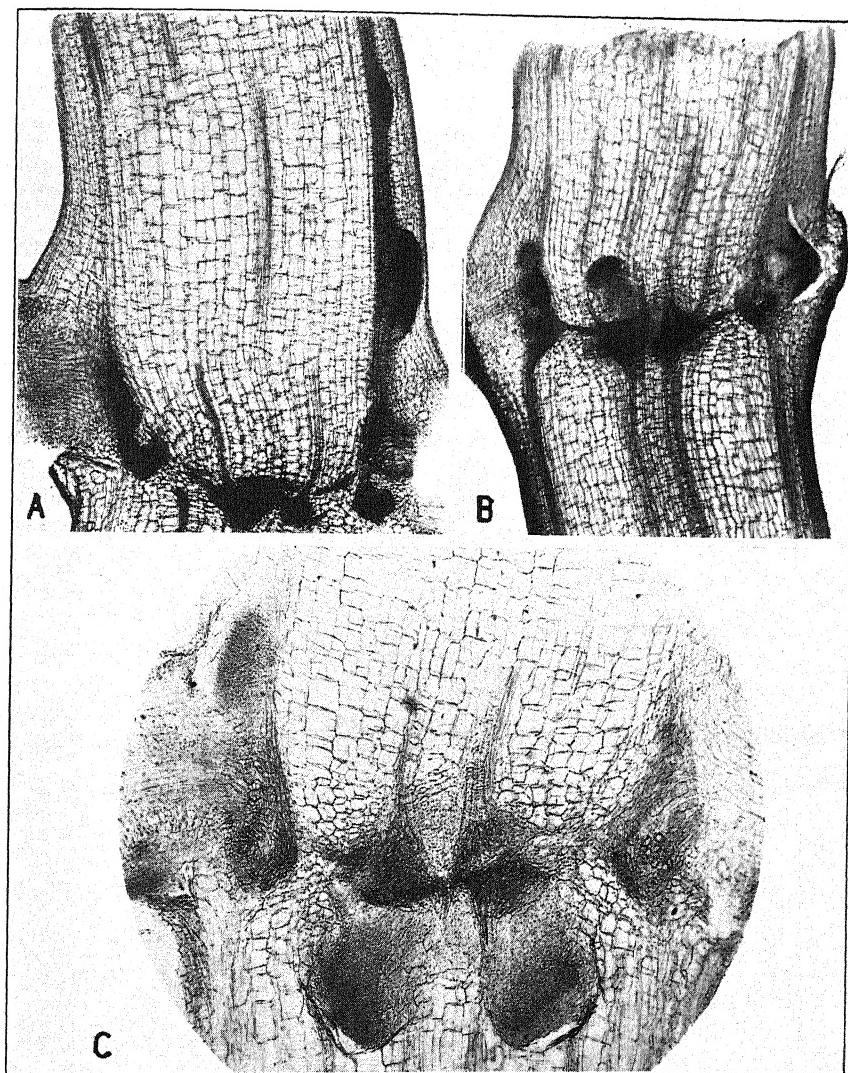
FIGURE 3. Longitudinal sections of treated nodes and internodes. (A) Node I treated with 0.5 per cent indolebutyric acid from May 13 to May 24 showing bases of two big roots, formed from the nodal peripheral meristem (roots cut off) and two internodal root primordia outside a peripheral bundle. (B) Node I treated with 0.3 per cent naphthaleneacetic acid from May 25 to June 14, showing peripheral nodal roots and one root arising above the nodal plate and growing upward between the central pith bundles as in Figure 2 B. (C) Node O_1 treated with 0.3 per cent naphthaleneacetic acid from May 13 to May 24, showing origin and vascular connections of large nodal root plates, a peripheral root forming slightly above (left), and meristematic activity above and below the nodal plate which has led to formation of two roots growing downward into internode O_1.

Effect of wounding. Below wounded parts of internodes, nodes, and leaf sheaths of varying age, effected by cutting away lateral strips of tissue or by oblique cuts into the internode only, a vigorous wound meristem is formed and the wound effects spread over a certain area in which they induce various anatomical and microchemical changes previously described (1). The phloem and sheath cells of the peripheral bundles around the wound edge and of the internal bundles abutting on the wound surface are especially active in contributing to the wound tissue. If such wounded regions are treated with growth substance paste, in addition to these changes root formation is stimulated in the usual manner in nodes toward the base of the stem, but this activity shows a striking increase within the area abutting on the wound. Root formation is promoted also from the node above the wound, and around the edge of the wound activity of the peripheral bundles leads to formation of numerous root initials or roots. Cross sections through the internode frequently show roots formed from cortical, perimedullary, and medullary bundles also.

Comparable changes occur in shoots which are decapitated below node I or above node II. Both in the treated plants and in the controls a cork barrier is formed below the cut surface, and the wound effects spread downward in the particular manner previously described (1, 2) by means of the intercellular system or along conducting passages, e.g., the wide xylem channels, in which necrotic changes occur and which also become blocked by tyloses formed from the surrounding living cells (Figs. 2 C, 4 A). No roots were formed in the controls, but their formation was greatly stimulated in the treated plants. Below the cut surfaces roots appeared first from the peripheral bundles (Fig. 4 A), and farther below from the internal bundles (Fig. 2 C), often occurring as far down as the nodal plate. It was interesting to note that in regions where the large xylem channels of the bundles became necrotic or blocked by tyloses the phloem remained intact and contributed to the newly forming roots. The same processes occurred in the longitudinal veins of the thin leaf sheath fragments surrounding the base of the decapitated internode. This includes root formation, as was shown by examination of leaf sheath II. Similarly isolated leaves, the bases of which had been treated with naphthaleneacetic acid paste, formed roots readily from the leaf sheath bundles.

Cork formation and tissue proliferation were generally small in intact plants, and in wounded ones not much larger than usual. In areas of nodes and internodes abnormally swollen owing to treatment, the cells of the superficial layers of epidermis, collenchyma, and cortical parenchyma showed very regular radial stretching and tangential divisions, thus forming a kind of superficial cork normally not occurring. This condition is not infrequently seen in the abnormally short and thick internodes formed under the treatment, as far as internode O_3 or O_4, but though there is a
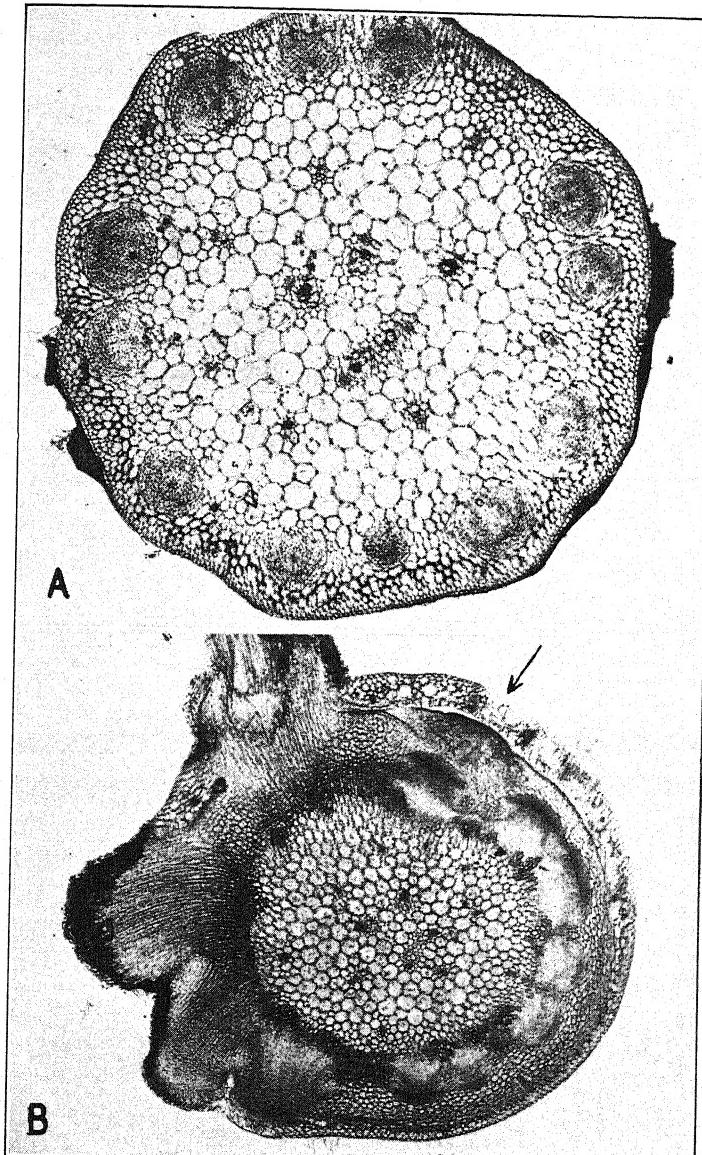
FIGURE 4. Transverse sections of treated nodes and internodes. (A) Internode I decapitated about 2 mm. below node I and surface covered with 1 per cent indolebutyric acid, showing tyloses and necrotic deposits in xylem channels of pith bundles about 2 mm. below wound surface and 12 root primordia originating from peripheral bundles. Section at a slightly higher level than Figure 2 C. (B) Asymmetrical root formation in node I after treatment with indolebutyric acid from February 10 to March 30, showing outside the ring of peripheral bundles seven root initials, one is located farther inside opposite the suppressed axillary bud (see arrow); note large fasciated roots arising on the lower side of the node.

tendency in certain regions of the cells to extend radially rather than longitudinally and to divide in the tangential plane, further, more detailed inquiries will be necessary as to how the cells which are in the vacuolating and dividing stage are affected by the treatment.

In isolated treated leaves the large epidermal cells became hyperplastic and formed irregular outgrowths; it is probable that this layer becomes affected in the bending reactions of the leaves and leaf sheaths. Further investigations are necessary as regards unilateral growth reactions of the stem, which could be induced when growth substance paste was applied on one side to a node or internode of vertically growing shoots which had lost their natural dorsiventrality after three to four weeks in the dark room.

DISCUSSION

The general nature of the various responses of *Tradescantia fluminensis* to treatment with synthetic growth substances is analogous to that described for dicotyledons and evidently has a correlation with the structural and developmental features of the plant. In *Tradescantia*, one common representative of the monocotyledon growth type, the separation of the leaf primordia by internodal extension commences a short distance behind the growing point and later this elongation process is characterized within the different segments of the shoot by the gradual basipetal disappearance of a peripheral meristematic cylinder situated between cortex and medullary region. Meristematic activity is continued longest in the node where vascular differentiation is still taking place both in the peripheral and medullary region and where there is formed an axillary branch and peripheral adventitious roots develop very gradually and lie dormant until suitable conditions for their further development arise. Furthermore there are present throughout the internode certain pericyclic layers of cells, both in the peripheral region and around the various internal bundles, whose cells, perhaps in conjunction with activities in the phloem, can be induced to return more readily than others to the meristematic stage, as shown by their behavior in the wound healing processes and during vegetative propagation in cuttings. This pattern of meristematic and potentially meristematic zones characteristic for the adult internode is already fully indicated in the extending and vacuolating segment beneath the third primordium from the apex, and here chemical stimulation has the effect of accelerating and modifying certain developmental processes, in particular of initiating prematurely the formation of roots. In the older part of the stem such stimulation is still effective in the more meristematic parts of the node and in other regions as far as advanced tissue specialization permits cells to return from the vacuolated stage to engage in such activity. The older the segment the less vigorous and irregular in shape are the roots formed from the basal remainders of the peripheral meristem, but

internodal roots may still be comparatively readily formed outside the peripheral bundles which are late in becoming differentiated and the xylem of which does not become ruptured like that of the internal bundles; roots may also be formed around the internal bundles in the more meristematic region above and below the nodal plate.

Numerous observations recorded in the literature indicate that meristematic activity and general regeneration and growth processes are often linked up with the presence or differentiation of phloem tissue; phloem cells may contribute to the wound tissue themselves (1) and they have been found responsive to growth substances in dicotyledons where rapid anatomical changes were noticed in the phloem as early as in the pericycle (7, 9, 10, 13). In the present investigation they were found to become affected by the treatment and to be actively engaged in the root forming processes both in the peripheral and internal bundles. In decapitated or wounded portions of internodes or of leaf sheaths the xylem parts of the bundles became necrotic and blocked by tyloses while root formation proceeded in conjunction with phloem activity as usual. On the other hand, the vessel and tracheid systems of intact leaves, internodes, and nodes were also often visibly affected in a peculiar manner, containing, greyish dense masses, which stained red with Sudan III, in portions of the shoot externally treated with growth substance lanolin paste.

In intact plants root formation after application of paste to aerial parts of both young and older portions of the shoot was promoted or induced for a considerable distance down the stem. In wounded segments roots were formed from the node above the point of application and particularly in the wounded internode itself within an area in which to a lesser degree anatomical changes took place after wounding only. The same groups of cells which are especially reactive in wounded internodes, either participating in wound cork formation or resuming and continuing at an increased rate normal developmental processes, such as wall thickening and lignification (1), engaged in root formation after chemical treatment.

Meristematic activity and root formation thus appear closely correlated with the original distribution of meristematic or potentially meristematic cells. In the node such cells are arranged asymmetrically; consequently both natural and stimulated root formation are asymmetrical. At present almost nothing is known about the developmental factors which control the differentiation and maintenance of such cells in definite positions in the plant. Differences in the reactivity of the cells cannot be discussed at their full value, until more adequate facts are available concerning the distribution (8), the manner of activation, and the reaction of the stimulating principle itself in the various cells and parts of the tissue. In *Tradescantia*, application of growth substances near the

apex in comparatively high concentration has a delaying effect on the developmental processes which take place in the terminal meristem and in the youngest still largely meristematic primordia. An analogous behavior is known for the vegetative point of roots. This illustrates the fact that cells and cell complexes which are very sensitive and exhibit the maximum regenerative power in processes of wound repair and direct regeneration, may show only limited responses when they are overstimulated with growth substances. The development of the axillary buds was also suppressed.

SUMMARY

1. Application of synthetic growth substances in comparatively high concentration in lanolin to the intact stem of *Tradescantia fluminensis* induces responses and histological changes generally comparable to those reported for dicotyledons, though somewhat less vigorous, viz. epinastic bending of leaves, meristematic activity, and production of roots, gradually decreasing toward the base of the stem.

2. In particular the reactions are related to the structural and developmental features of the plant as expressed in the pattern of meristematic and potentially meristematic cells distributed in the various regions of the monocotyledon shoot. Three regions may be distinguished, gradually passing over into one another, in which externally supplied relatively high concentrations of growth substances induce different changes: A. In the meristematic apex and the two youngest still largely meristematic primordia development is delayed. B. In the rapidly elongating portion of the shoot short and thick segments are formed in the basal parts of which differentiation processes are initiated prematurely and more vigorously than normal, resulting in the formation of numerous internal and fasciated external roots and of nodal vascular elements. C. In the nearly mature and mature part of the shoot root initials already present are stimulated and a great number of new roots are formed both in the node and internode.

3. Generally roots or root primordia are most readily formed from the peripheral and medullary meristematic zones of the node, and in the second place in the internode from corresponding layers outside the peripheral bundles, which are late in becoming differentiated and the xylem of which does not become ruptured. Roots are also formed from the internal bundles, especially above and below the nodal plate. In highly stimulated internodes all three origins of roots occur together. Root formation is symmetrical in the internode and asymmetrical in the nodal region.

4. Roots are generally formed outside the phloem regions which appear to be themselves responsive to the treatment. The xylem elements of intact lanolin-treated parts of the shoot are frequently filled with masses giving fat reactions.

In decapitated internodes and leaf sheaths the xylem channels become

necrotic and blocked by tyloses, while roots are formed outside the phloem of all series of bundles.

5. With the exception of the meristematic apical region, cells and tissues which are especially responsive to wounding are also very sensitive to growth-promoting substances. This is particularly the case with cells situated outside the peripheral and internal bundles. Normally these cells remain parenchymatous. They resume activity in internodes which are either wounded or treated with growth substance. The former case is characterized by cell growth and cell division or the differentiation of a lignified sclerenchymatous sheath, the latter by the formation of external and internal roots. The effect of wounding and simultaneous application of growth substances is two-fold: beneath the wounded part a meristem is formed and secondary growth processes and necrotic changes take place, while within the area of the wound root formation is stronger than in corresponding intact treated internodes, particularly from peripheral and other bundles abutting on the wound.

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THE COMBINED EFFECT OF LIGHT AND GRAVITY ON THE RESPONSE OF PLANTS TO GROWTH SUBSTANCES

P. W. Zimmerman and A. E. Hitchcock

A recent publication showed how tropic responses involved unequal growth rates on opposite sides of stems and how growth substances applied to distal parts of the stem caused abnormal curvatures indicating unequal distribution of the substance (5). The literature on tropisms involving the hormonal theory was extensively reviewed and needs no further mention in this paper. There is a generally accepted theory supported by many experimental facts that natural growth hormones in stems may become unequally distributed due to unilateral illumination or geotropic stimulation. In the case of light the growth hormone is redistributed in favor of the shaded side causing positive phototropism. In case of horizontal stems the natural growth substance is redistributed in favor of the lower side where it accelerates growth, causing negative geotropism.

Synthetic growth substances applied to one side of a stem accelerated growth locally and caused curvatures resembling natural phototropism and geotropism (4). If applied to the upper side of a stem immediately upon being placed in a horizontal position, the synthetic substances caused downward bending. If applied to one side of an upright stem in good light or in the dark, growth was accelerated on the treated side and negative curvature resulted.

If, however, natural phototropism or geotropism or both occurred before the synthetic substance was applied, the effects were greatly modified. The purpose of this paper is to extend the first report (5) and to show how the combined effect of phototropism and geotropism condition the capacity of plants to respond to growth substances.

METHODS AND MATERIAL

Tomato plants (*Lycopersicon esculentum* Mill.) five to ten inches in height and grown in four-inch pots constituted the main plant material. The three most effective growth substances, naphthaleneacetic, indoleacetic, and indolebutyric acids, were mixed with lanolin and used as preparations which were applied with a glass rod unilaterally to the stems. The percentages of the substances used in lanolin ranged from 0.1 to 1.0 per cent. Generally 0.1 per cent of naphthaleneacetic acid and indoleacetic acid and 0.5 per cent of the indolebutyric acid were most satisfactory. To bring out the effects of phototropism and geotropism the plants were placed in various positions in an enclosure made of black cloth and open

on one side to permit of unilateral illumination. Within the enclosure the plants were placed upright, horizontal with the tip pointing toward the light, and horizontal with the tip pointing away from the light. In some cases plants were placed on a bench in the laboratory and the window served as the source of unidirectional light.

Some of the plants were treated with the substances at the beginning of the experiment before tropic responses could occur. Others were allowed to make the normal tropic responses before the growth substances were applied.
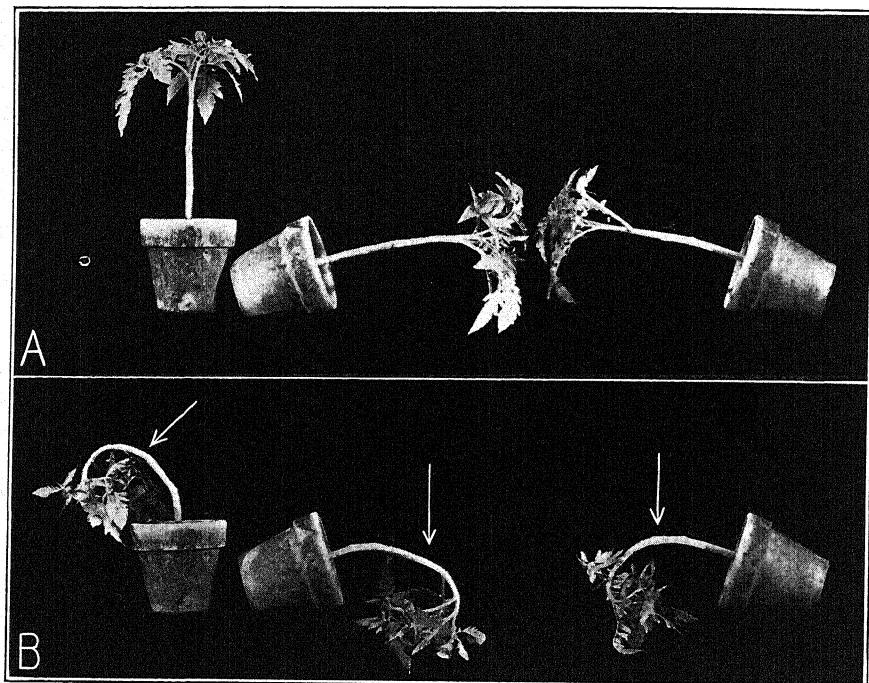
FIGURE 1. The response of tomato plants treated along one side of the stem with 0.25 per cent lanolin preparation of indoleacetic acid while in an enclosure with light from one side only. The light source was from the right side. A. Appearance of the plants at the beginning of the experiment. After this photograph was taken the plants were placed in the enclosure and treated at once, as indicated by arrows. B. Appearance of plants six hours after treatment. Upright plant was treated on light side; horizontal plants were treated on the upper side. There was no apparent effect from unilateral lighting in this case.

RESULTS AND DISCUSSION

Plants treated with growth substances immediately upon being placed in unilateral light while upright or in a horizontal position responded similarly to those treated in the dark or in the greenhouse where light came from all sides. Growth was accelerated on the treated side of the
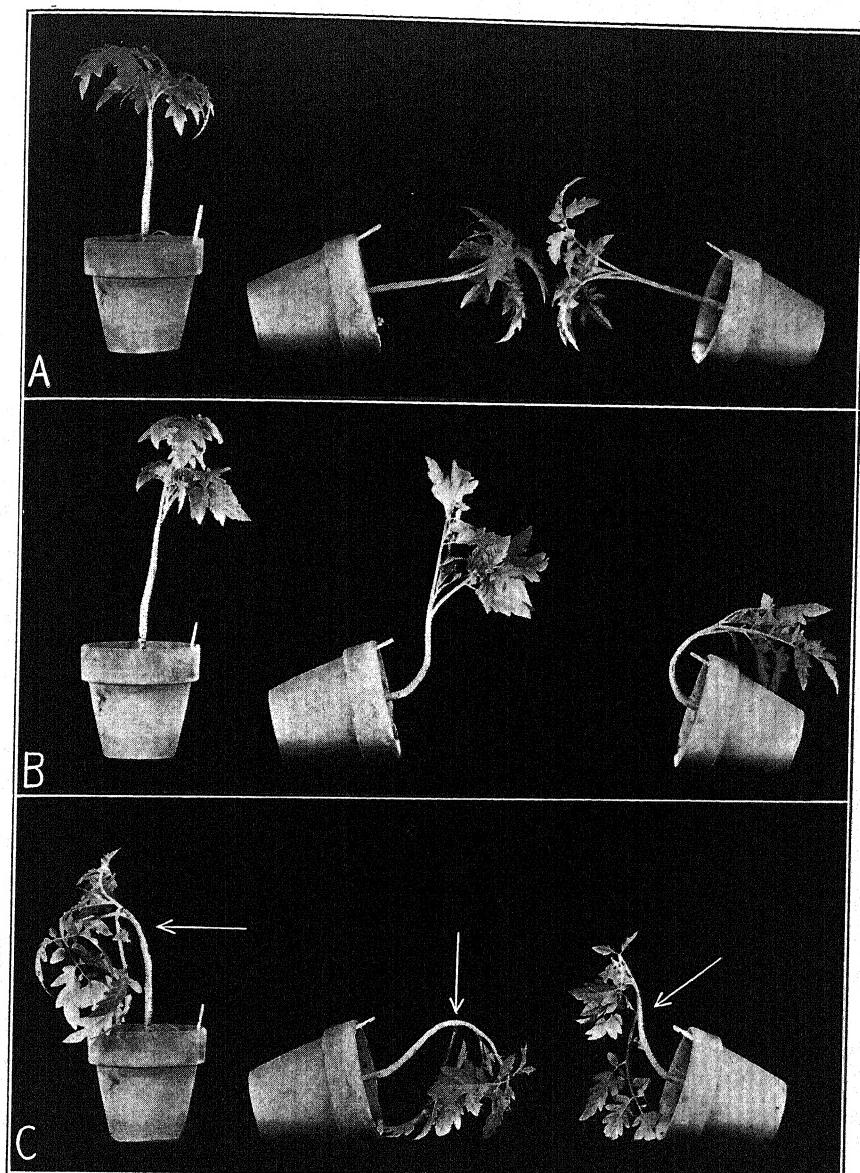
FIGURE 2. The influence of light and gravity on the response of tomato plants to 0.25 per cent indoleacetic acid. Plants were placed in an enclosure after the first photograph was taken. The light source in all cases was from the right side. A. Appearance of plants at the beginning. B. Same plants 24 hours later. Note combined effect of light and gravity. C. Same plants six hours after treatment along one side (see arrows). Upright plant was treated on light side. The two horizontal plants were treated on the original upper side. Compare with Figure 1 B. Note the effects of previous natural responses.

stem, inducing curvatures (negative) with little or no respect to light or gravity. Figure 1 shows a set of plants which illustrates the results. It was assumed that the synthetic substance applied to one side of the stem became effective before the natural hormones could be redistributed from the force of gravity or unilateral lighting.

Plants which were allowed to respond to gravity and unilateral light for 24 hours before being treated with the synthetic substances did not respond like the first set. From the results it was assumed that the natural hormones had been effectively redistributed in favor of the shaded and lower sides of the stems. The response of the plants is illustrated in Figure 2 B. There appears to have been a combined effect of light and gravity in the horizontal plants. The one pointing toward the light first responded to the force of gravity. As the stem turned away from the earth supposedly due to accumulation of substances on the lower side the force of unidirectional lighting came into play working in opposition to the first unequal distribution of the hormone.

The other horizontal plant shown in Figure 2 B pointing away from light also shows a combined effect of gravity and light but in a different way. The lower side of the stem was first stimulated through the force of gravity and as it grew away from the earth, the original lower side became also the shaded side. It thus had the added advantage of unilateral light. The combined effect of light and gravity induced a pronounced curvature.

Figure 1 shows the effect of treating plants on one side of the stem before the forces of light and gravity became effective. Figure 2 C illustrates the results of first allowing the natural forces to come into play before the synthetic substances were applied. The lanolin preparations were placed along the light side of the upright plants and on the sides which were up when the other two plants were first placed in a horizontal position. The photograph was taken six hours after the chemical treatment.

The horizontal plants showed definite effects of the chemical preparation but the response was conditioned by the earlier phototropism and geotropism. The plant pointing toward the light was treated on the dark side (after geotropic response) where the natural hormone is supposed to accumulate and the effect was additive, causing downward growth. In the other plant the light side of the stem was treated and, if the assumption is correct, the synthetic substance was opposed by the natural substances which had accumulated on the dark side.

The upright plant curved somewhat when treated on the light side, but the response was much less than when similar stems were treated on the light side at the beginning of the experiment before the natural forces became effective (Fig. 1 B). In other experiments where stems of upright plants were treated on the shaded side the degree of response was greatly increased. Similarly a plant pointing away from light, as in the case of the

one on the right, Figure 2 C, showed a pronounced additional response when the stem was treated on the shaded side. When the synthetic substance was applied to the side of the stem where the natural hormones were assumed to accumulate due to phototropic or geotropic stimulation, the degree of response was greater than with comparable stems which had not been previously stimulated.

Geotropic stimulation alone is sufficient to retard the degree of response when stems of horizontal plants are treated on the upper side. This fact was first observed when motion pictures were being taken to show induced tropic responses of horizontal plants from unilateral treatment of the stems with growth-promoting chemicals. Plants treated on the upper side of the stem immediately upon being placed in a horizontal position curved downward 90° or more. Plants which had made a geotropic response before treatment moved down only to the horizontal position. In the latter case, apparently the synthetic substance had to work in opposition to the natural substance which had accumulated on the lower side of the stem. Measurements of growth made by surface markings showed that when natural geotropic response occurs the amount of growth on the lower side of the stem was 10 to 20 times as great as on the upper side (5). When geotropically stimulated stems were treated on the upper side growth was stimulated and continued until it equaled that of the lower side. This fact accounts for the failure of the geotropically stimulated stem to bend beyond the horizontal position when treated on the upper side with growth-promoting substance.

The assumptions concerning unequal distribution of natural and synthetic growth substances in geotropically stimulated stems are supported by the results of recent experiments designed to recover indole compounds applied to distal parts of stems. Excised tomato shoots were placed in a horizontal position and treated at the basal end with 50 to 100 mg./l. of indolebutyric acid. As soon as a definite growth response (curling and epinasty of leaves) occurred (2 to 4 hours) the leaves were removed and the stems divided into upper and lower portions. These were frozen with Dry Ice (CO_2) and 32 grams of each used according to the suggestion of du Buy (1) for extracting natural substances. The tissue was ground while frozen with 25 cc. of slightly acidified water (1 cc. of acetic acid per liter of water) and filtered through a Buchner funnel. An excess of water (about 50 cc.) was used to further extract the tissue in the filter. The filtrate was then boiled down to 50 cc. and extracted for 24 hours with 55 cc. of ether. The ether layer was then separated and evaporated to dryness without heat. The residue contained the indole substance which gave a positive color test according to the Winkler and Petersen method (3). An effort was made to compare the amount of the substance in the upper and lower sides. For this purpose the colorimeter, the dilution method, and the biological

test for epinasty were used. The colorimeter indicated three times as much indole substance in the lower as in the upper sides. The dilution methods verified the colorimeter tests. For the biological test excised shoots of young tomato plants were placed upright with the basal ends in the solution. Both solutions induced epinasty of the leaves but the extract from the lower side of the stem induced the more pronounced response. The extracts from water control plants gave negative results in all cases. It appears, therefore, from these results that the synthetic growth substance was unequally distributed in horizontal stems in favor of the lower side. It cannot be said at this time that the extracted substance was the same as that applied. The extracted substance, however, was physiologically active (induced epinasty), and the color reaction strongly resembled that of indolebutyric acid which was applied to the base of the shoots. These facts suggest that the indolebutyric acid molecule might have remained intact. Similar experiments have been conducted, also, with indoleacetic acid.

Two earlier reports (4, 5) showed that small tomato plants, placed in the dark four to five days, lost their capacity to respond to the force of gravity. When growth substance was applied laterally to the stems of these depleted plants, negative curvatures occurred. Also, if water solutions of the substances were applied to distal parts of these plants, they regained their capacity to respond to gravity. From these results it was assumed that plants failed to respond after they lost their natural growth substances in the dark, and that the synthetic substances could be substituted at least in part.

Oortwijn Botjes (2, p. 7) repeated these experiments and made the following statement: "The results obtained with this investigation prove the hypothesis of Zimmerman and Hitchcock that tomato plants lose their growth substance in the dark." This author further showed that after the plants lost their capacity to bend when placed in a horizontal position the shoots could still be polarized by gravity. This polarization influenced the direction of bending when growth substance was finally applied.

It seems from the results to date, that the next logical step should be to test the depleted plants in unilateral light while supplying the synthetic growth substances in various ways. Such plants might be also good material to study the capacity of tissues to distribute growth substances according to the stimulus applied. In this connection the new method for detecting traces of indole derivatives in plants is sure to prove useful in growth substance research.

SUMMARY

To account for natural tropic curvatures, the assumption was made that natural growth substances were unequally distributed during phototropic and geotropic stimulation.

Stems treated unilaterally with lanolin preparations of growth substances made pronounced bending responses when in the dark or when light came from all directions. This was true also of stems treated immediately upon placing the plants in a horizontal position or in unidirectional light.

If plants were allowed to make natural tropic responses before the synthetic substances were applied, the induced responses were greatly modified. When chemical substances were applied opposite the side natural hormones were assumed to accumulate, the degree of response was greatly reduced; applied on the other side the response was greatly accelerated. In the first case, it was assumed that the synthetic substance worked in opposition to the natural hormones and in the latter case with them.

There was a difference in natural response when plants were placed in a horizontal position while in an enclosure with unilateral lighting. After making a natural response these plants showed a combined effect of light and gravity when treated on the original upper side of the stem, presumably due to unequal distribution of natural substances first caused by the force of gravity and then by light.

Synthetic growth substances applied at distal parts of tomato stems were extracted and identified by the colorimetric method as indole derivatives. Indolebutyric acid applied to the basal end of a horizontal stem was unequally distributed, more going to the lower than the upper side. The extracted substance was not definitely identified as indolebutyric acid. but it was physiologically active (induced epinasty) and the color reaction strongly resembled that of the acid which was applied to the base of the shoot. The facts suggested that the indolebutyric acid molecule might have remained intact in the tissue.

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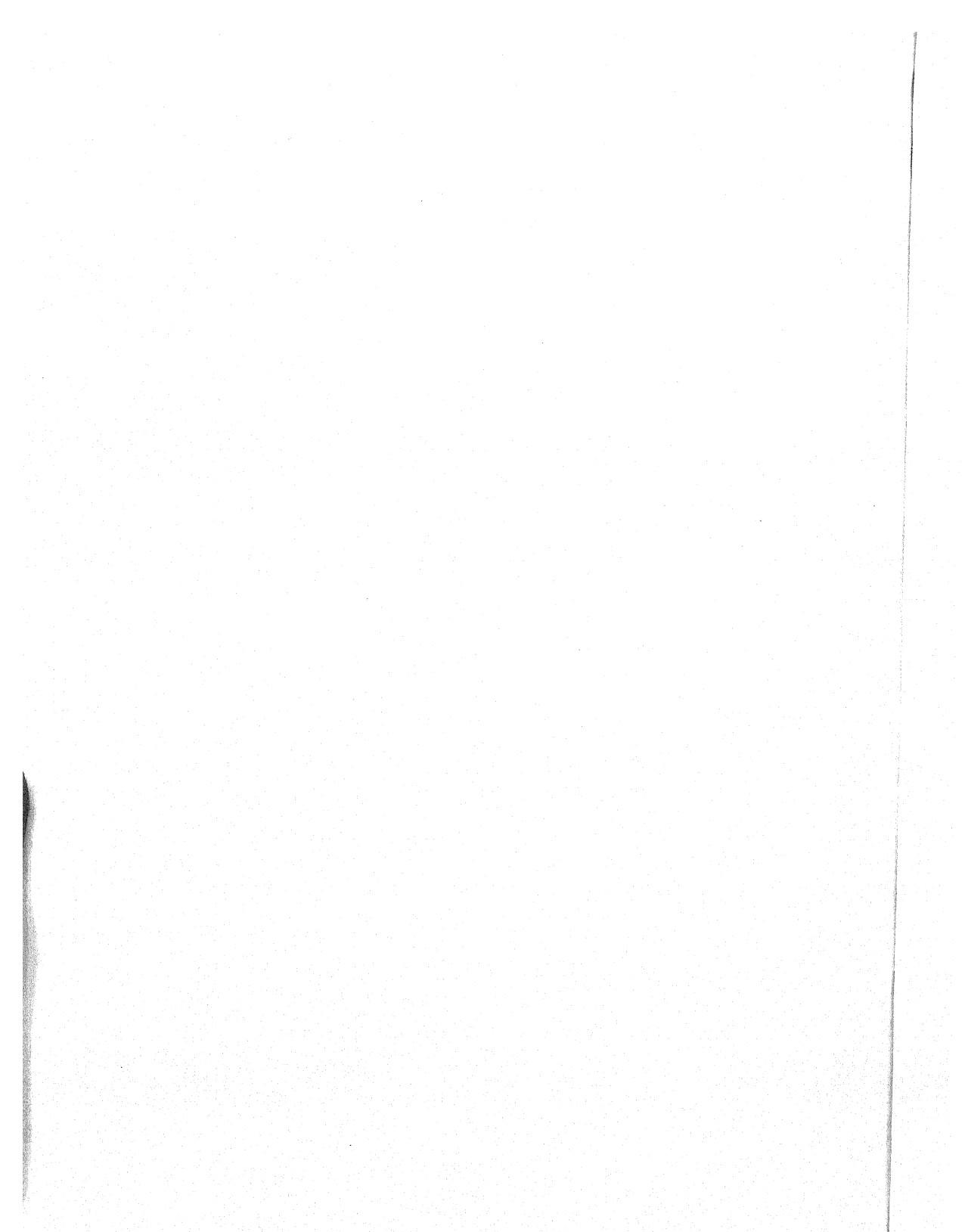
THE USE OF GREEN TISSUE TEST OBJECTS FOR DETERMINING THE PHYSIOLOGICAL ACTIVITY OF GROWTH SUBSTANCES

A. E. HITCHCOCK and P. W. ZIMMERMAN

Results obtained in our laboratory during the last few years have indicated that explanations for growth promotion and root formation in special test objects such as the *Avena* coleoptile, and hypocotyls of the pea, bean, sunflower, etc., fall far short of explaining what occurs in normal plant tissue and in tissue treated under natural conditions with growth substances. Reference to the literature on the action of growth substances in plants shows that more emphasis has been placed on the necessity for rigid adherence to some of the earlier views, on purely theoretical grounds, than on modifying them so as to be in accordance with the facts at hand. In such a rapidly advancing field it is not unusual that there should be differences of opinion relating to the interpretation of certain data, but as additional data accumulate, it is obvious that such information should not be excluded merely because it fails to agree with previous veiws. Assumptions which were originally used as an aid in the formulation of working hypotheses have too frequently been regarded as proof for supporting certain views. This has led to the use of explanations which rest so largely on assumptions that changing or rejecting one will also affect many others. Under these circumstances there may be a tendency to seek additional special cases which would appear to support a given view rather than to give a fair consideration to all facts, particularly those opposed to the view. It is the purpose of the present paper to discuss such examples and to compare the results reported for special test objects with those obtained with normal plant tissue treated under natural conditions, particularly with reference to root formation. Special attention has been devoted to an analysis of published data used in support of views relating to the specific action of growth substances in the higher plants. Much of the data in the present paper appeared earlier in abstract form (44, 45, 46, 47).

In view of the disagreement between the explanation of results obtained with special test objects and those obtained with normal plant tissue taken from plants growing under natural conditions, it seemed of interest to select a sensitive test object for root formation studies which is representative of cuttings used in commercial practice. Although the privet and certain other varieties have been used as standard test objects for studying root formation in cuttings of woody plants (124, p. 346), it was desirable to select more sensitive plant material rooting in a much shorter time. The selection of the tomato leaf was based on several considerations.

Treated cuttings rooted in five to seven days whereas few or no roots had emerged from control cuttings in this time. The test method itself is extremely simple and is economical with respect to time, labor, and cost of equipment and materials. Of particular interest is the fact that the tomato leaf is highly sensitive and hence requires the use of relatively low concentrations (0.01 to 3.0 mg. per liter) of growth substance. Not only is this test more sensitive than others previously reported for root formation, but it compares favorably with the most sensitive tests for growth promotion.

Results obtained with the tomato leaf confirm in general those previously reported (38, 42, 124) with respect to the relative activity of different substances, the relation between activity and the form of the substance (acid, salt, or ester), relationships between the type of material and root-forming activity, the influence of the solvent, and the upward movement of the basally applied substance. All of these results point toward the desirability of considering the action of growth substances from the standpoint of what occurs under natural conditions in normal plant tissue as well as what occurs in special test objects under highly restricted conditions. A number of explanations will continue to remain questionable so long as they rest solely or mainly on special cases. The basic procedure whereby one factor at a time is varied while the influence of all others is suppressed or eliminated, is important and useful, but the true evaluation of several important interactions is not always readily obtained in this manner. Our use of normal green tissue for test objects has been criticized (34, p. 853; 99, p. 911; 114, p. 55; 116, p. 51) but we wish to point out that through this path of approach to the problem it has been possible to distinguish between factual evidence which is fundamental for green tissue in general, and evidence which applies mainly to etiolated tissue. Against the normal background of complex interactions in our test objects we have been able to demonstrate the relative importance of a number of different factors for root formation which it has been impossible to accomplish by the use of etiolated tissue. It is our belief that by the methods developed in our laboratory and similar ones developed more or less independently by Laibach and his associates, it has been possible to change the trend of growth substance research from a highly theoretical plane to one in which the principal interest is centered in dealing with facts in relation to normal tissue. The terms green tissue or normal tissue are used throughout this paper to designate pigmented tissue in contradistinction to etiolated tissue which lacks the pigments that are normally formed when the tissue is exposed to light.

MATERIALS AND METHODS

The three principal growth substances (indoleacetic, indolebutyric, and naphthaleneacetic acids, and their salts and esters) used in the present experiments were obtained from Merck & Co. Inc., Rahway, New Jersey.

Freshly prepared solutions were used for each test. Leaf cuttings of Bonny Best and Marglobe varieties of the tomato (*Lycopersicon esculentum* Mill.) were the principal test objects.

Besides the tomato, cuttings of the following species (including two or more varieties in several instances) were used in the root formation tests: *Acer palmatum* Thunb., *Ageratum* sp., *Arctostaphylos Uva-ursi* Spreng., *Azalea obtusa* Lindl., *A. mollis* Blume, *Berberis thunbergii* DC., *Buddleia davidi* Franch., *Buxus sempervirens* L., *Callicarpa japonica* Thunb., *Camellia japonica* L., *Catalpa hybrida* Spaeth., *Celastrus orbiculatus* Thunb. (*C. articulatus*), *C. scandens* L., *Chamaecyparis obtusa* Sieb. & Zucc., *Chrysanthemum* sp., *Clematis* sp. (*lawsoniana* hybrids), *Coleus blumei* Benth., *Cornus kousa* Buerg., *Corylus avellana* L., *Cotoneaster racemiflora* Koch., *Cryptomeria japonica* D. Don., *Dahlia variabilis* Desf., *Daphne cneorum* L., *Deutzia magnifica* Rehd., *Dianthus caryophyllus* L., *Diervilla hybrida* Dipp. (*Weigela*), *Euonymus radicans* Sieb., *Forsythia suspensa* Vohl., *Gardenia* sp. var. Hadley, *Gordonia alatamaha* Sarg., *Halesia carolina* L., *Hedera helix* L., *Hibiscus syriacus* L., *Hydrangea radiata* Walt., *Ilex aquifolium* L., *I. cornuta* Lindl., *I. glabra* Gray, *I. opaca* Ait., *Juniperus sabina* L., *Kolkwitzia amabilis* Graebn., *Ligustrum ovalifolium* Hassk., *Lonicera maacki* Maxim., *Magnolia soulangeana* Soul., *Malus niedzwetzkyana* Aschers & Graebn., *Morus alba* L., *Pachysandra terminalis* Sieb. & Zucc., *Petunia hybrida* Vilm., *Philadelphus virginalis* Rehd., *Prunus triloba* Lindl., *Pyrus malus* L. var. Rhode Island Greening, *Rosa* sp., *Sambucus canadensis* L., *Spiraea bumalda* Burv., *Syringa vulgaris* L., *Taxus cuspidata* Sieb. & Zucc., *Viburnum opulus* L.

Species and varieties other than those listed were also used, particularly in the case of *Rosa, Chrysanthemum, Camellia, Viburnum, Spiraea, Deutzia, Berberis, Dahlia,* and *Syringa*. Horticultural varieties of *Syringa vulgaris* were taken the latter part of April and during May. Most deciduous shrubs were taken at this time, as well as at later periods.

A sufficient amount of the growth substance solution to supply the cuttings for 24 hours was placed in beakers or flasks of 50 to 150 cc. capacity. The basal ends of the cuttings were immersed to a depth of 2 to 3 cm. in the test solution. At the end of 24 hours the cuttings were removed from the test solution, washed, then placed with their basal ends in tap water for the remainder of the test period. Roots emerged from the treated tomato leaf cuttings on the fourth or fifth day. The root counts were made on the sixth or seventh day depending upon the relative activity of the cuttings and the atmospheric conditions. Cuttings of other species of plants were treated in the same manner for the first 24 hours, but were handled thereafter according to methods commonly used by propagators. The time at which root counts were made in this case depended upon the species of cutting, ranging from two to several weeks.

Leaf cuttings of the tomato ranged in length from 15 to 25 centimeters.

One to three leaves were taken from a single plant, depending upon its size and rate of growth. The youngest leaves at the tip (less than 15 cm. in length) and the older basal leaves were generally less satisfactory than the largest leaves on the upper part of the stem. In contrast to the leaves leaflets rooted less readily, and stem cuttings more readily. Thus different parts of the tomato plant may be used for root formation tests, depending upon the particular purpose of the test. The leaf was considered most satisfactory for the tests described in the present paper. As a matter of interest it should be mentioned that leaves of the tobacco (*Nicotiana tabacum* L. var. Turkish) were much more difficult to root than tomato leaves.

Leaves were severed at the base of the petiole except when otherwise specified. During the summer such leaves rooted more readily than when cut 1 cm. above the base. The reverse was true for leaf cuttings taken during the winter. Since leaves were more sensitive in the summer than in the winter, regardless of the position of the basal cut, it is preferable to sever the leaves 1 cm. above the base for tests carried out during the winter. In comparative tests it is important that the position of the basal cut should be the same.

Since the absorption of the test solution is influenced by atmospheric conditions (42), this fact should be considered when comparing results obtained at different times. All of the tests with tomato leaf cuttings were run in a Wardian type of glass case placed in the greenhouse. Relative humidity was kept high enough to prevent wilting by adjusting the width of opening in the front door. Tests with all other varieties of cuttings were run in a laboratory having an eastern exposure. In this case the cuttings were 5 to 10 feet from the windows during the 24-hour period of treatment with growth substance solution. Thereafter the cuttings remained where planted in a mixture of sand and peat moss in the greenhouse bench as previously described (43, p. 64).

Design of experiments. Most of the experiments were designed to study a number of factors simultaneously. Factorial experiments of this type are especially adapted to the determination of the relative importance of any one of several factors under conditions in which chance deviation of individual test objects is minimized. Intercomparisons of the performance of individuals within a group and between groups are contrasted against the background of the entire experiment. The interpretation of such complex experiments is actually more readily accomplished and represents a more precise evaluation than is the case where separate experiments are performed at different times. There is the additional advantage that, by the scheme described, all data are used, so that no opportunity is afforded for the selection of special cases to illustrate a particular point. The advantages of this type of layout of experiments, particularly for statistical treatment according to the analysis of variance, are described more fully in a paper by Setterstrom, Zimmerman, and Crocker (87).

The experimental scheme referred to permits of the arrangement of the results in a number of different groupings whereby the reader may observe at a glance any important trends which are reflected in column totals and group totals. A complex experiment such as that recorded in Table XIII requires a rearrangement of the same data for the sytematic study of different conditions by means of contrasting pairs (or groups) of test objects. It is to be noted that all data appear in Table XIII for 16 experiments comprising 12 tests each, or a total of 192 tests performed simultaneously. However, since in the present paper only general trends represented in the larger group totals are referred to in the text, a detailed analysis of the results of each experiment has been omitted. Nevertheless, the data are available for those who may wish to make such an analysis, particularly in the case of a complex experiment such as that recorded in Table XIII.

RESULTS AND DISCUSSION

Specificity of the action of growth substance. A number of substances previously found active for growth promotion and root formation on intact plants were tested for root-forming activity in leaf cuttings of the tomato. Results of these tests were in agreement with those previously reported (43, 124, 127) in showing that the indole and naphthalene compounds were more active than the phenyl compounds. Consequently, in the principal root formation tests the three most active substances were used—that is, indolebutyric, naphthaleneacetic, and indoleacetic acids, and certain of their salt and ester derivatives.

Tests with several phenyl derivatives indicated that they are active at concentrations of 1 to 10 mg. per liter for root formation in the tomato leaf. At concentrations of 1 to 10 mg. per liter the following substances were active, but less active than a solution of indolebutyric acid containing 0.032 mg. per liter: phenylacetic acid, phenylpropionic acid, sodium cinnamate, nitrocinnamic acid, and aminophenylacetic acid.

By regulating the conditions of the tomato leaf root formation test, it was possible to obtain root formation in cuttings treated with 10^{-6} M solutions of growth substances known to be active for rooting and no rooting in control cuttings or in cuttings treated with acetic acid or potassium acetate (Fig. 1 B). The young leaves for which data appear in Figure 1 B were taken from small plants four to five inches tall, whereas the data in Figure 1 A are for larger leaves taken from taller plants. In most tests, the type of material was selected which would show some but slight rooting in control cuttings at a time (sixth or seventh day) when treated cuttings had many roots. The specificity of the tomato leaf test depends, therefore, upon the condition of the tissue used for cuttings.

Considering that indolebutyric and naphthaleneacetic acids are more effective root-forming substances than any yet reported (127), it would appear that this constitutes the first case of a foreign substance being much
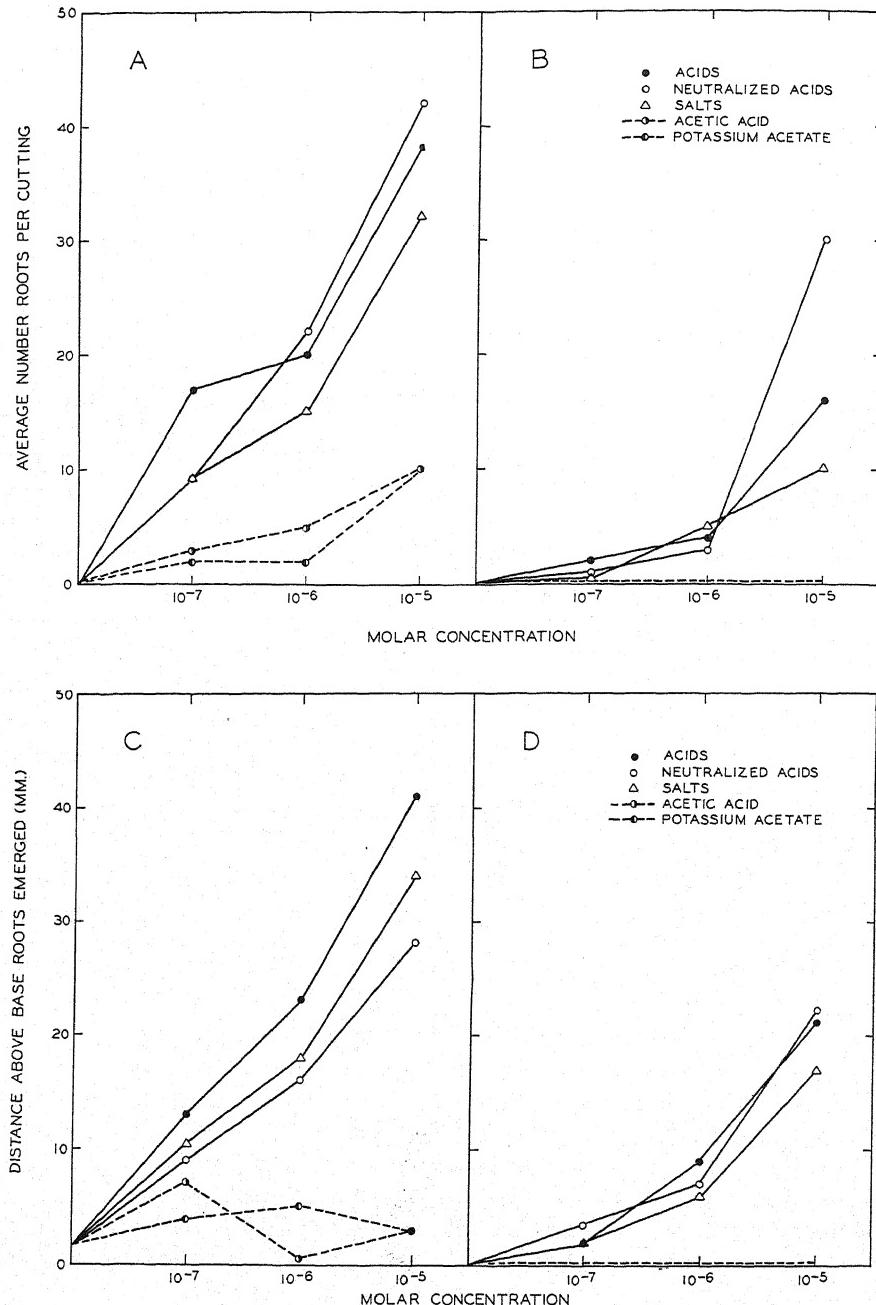
FIGURE 1. Curve showing root formation in tomato leaf cuttings treated for 24 hours with tap water solutions of growth substance. (A) Root counts on sixth day for large leaves, (B) same for smaller and younger leaves, (C) length of tissue from which roots emerged, for large leaves (seventh day), and (D) same for smaller and younger leaves.

more active than the naturally occurring hormone in plants, and also more active than either auxin A or indoleacetic acid. Limited data relating to the substances extracted from plant tissue (101) indicate that no naturally occurring substance is more effective for root formation than indoleacetic acid. Thus the high positions occupied by indolebutyric and naphthaleneacetic acids for root formation are similar to the high positions occupied by auxin A and indoleacetic acid for growth promotion in *Avena*. Indolebutyric and naphthaleneacetic acids are reported by Went and Thimann (116, p. 137) and Koepfli *et al.* (55) to be of the same activity as auxin A and indoleacetic acid for growth promotion in *Pisum*. Since physiological activity varies with the kind of test object, any specificity of chemical structure must also depend upon the kind of test object used.

That only a few substances may be active for cell elongation appears unlikely, considering the large number of substances reported as active for growth promotion in different test objects (5; 20; 55; 83; 116, p. 137; 120; 124). The same may be said of root formation if it can be shown as suggested by Traub (104) that many organic substances are active for root formation. It was not specifically mentioned how long the cuttings were treated and whether they were treated at the apical or basal end, but cuttings of *Passiflora* were treated in both ways. Traub's approximate method for designating the relative differences in rooting (plus signs) does not permit the reader to determine whether the claims for better rooting are justified. There is no reference to number of roots, length of roots, percentage rooted, or the length of tissue from which roots emerged—all of which are criteria used by other workers in such tests. The concentration of indolebutyric acid (0.02 per cent) which he used as a standard of comparison is exceptionally high for a 24-hour treatment and would be injurious to most of the varieties tested in our laboratory. On the basis of a 24-hour treatment, *Passiflora* and *Bignonia* would appear to be more tolerant and more difficult to root than any variety reported by us in which case it has seldom been necessary to use concentrations of indolebutyric acid higher than 0.004 to 0.006 per cent. Higher concentrations may of course be used for shorter time periods as in the case of apple (Fig. 2 A). Skinner (88) used 0.001 to 0.009 per cent solutions.

The assumption that there is a specificity of growth substance action in plants constitutes the basis for explanations relating to growth, differentiation, and correlation. It is significant, however, that no definition of a growth substance has been proposed which accounts satisfactorily for the facts at hand. Reference to reviews or any discussion dealing with the terminology or nomenclature of growth substances will show that there is agreement with known facts only to the extent that there is claimed to be in plants the counterpart of hormonal regulation in the animal body. The introduction of the term "auxin" for designating a particular kind of growth substance has been unfortunate, since it has been used in a specific sense
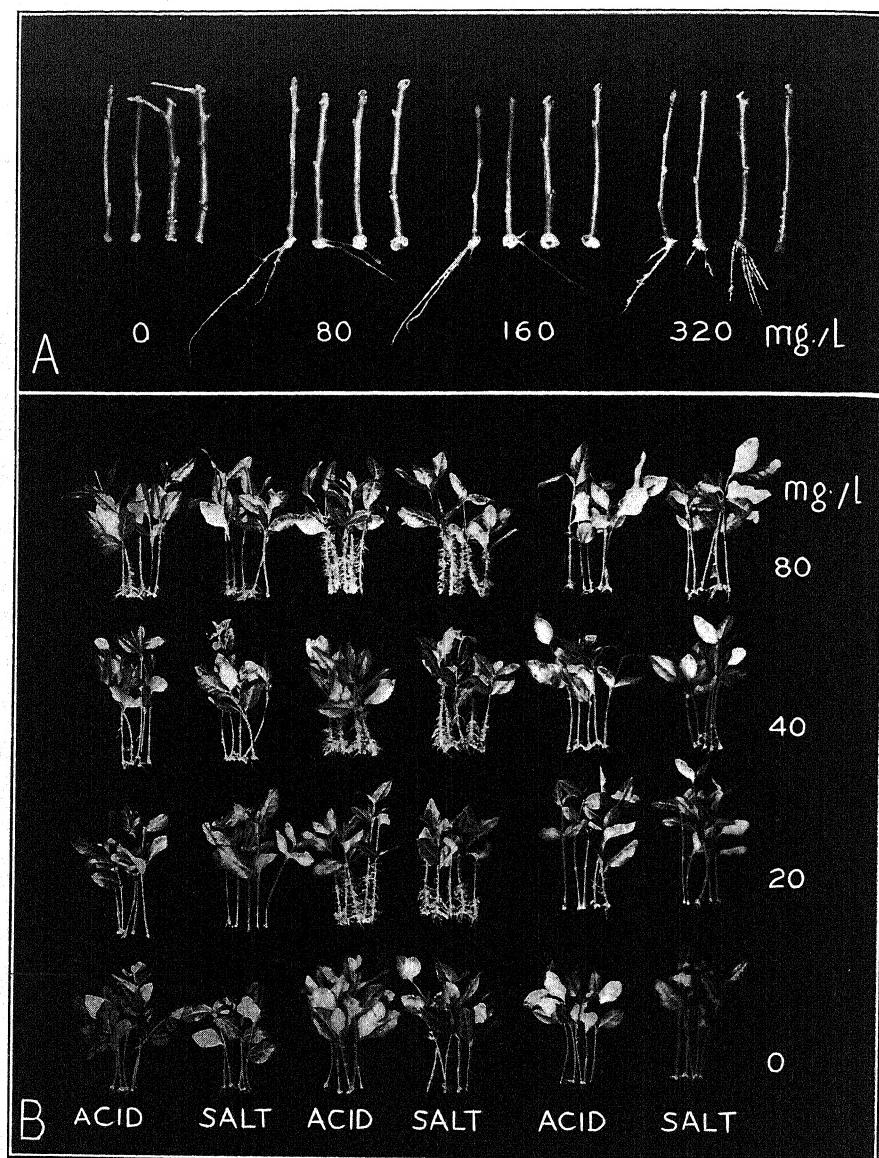
FIGURE 2. (A) Rhode Island Greening apple 38 days after a 4-hour treatment with indolebutyric acid. Tip cutting on left in each set; others taken below tip. Cuttings buried (after treatment) in moist moss peat in loosely covered glazed jars kept under the greenhouse bench. No water was added to the moss peat during the 38-day period. (B) Relative effectiveness of different growth substance acids and salts on root formation in *Euonymus radicans* (after 18 days). Let to right, acid and salt respectively of naphthaleneacetic, indolebutyric, and indoleacetic compounds.

to designate preparations known as auxin A, auxin B, auxin-A-lactone, heteroauxin, and also in the generic sense to designate known compounds, and also natural substances, which exhibit similar physiological responses. Koningsberger and Verkaaik (56, p. 10) have pointed out the undesirability of using "auxin" as Went and Thimann have done throughout their monograph on phytohormones (116) without designating what substance was used. The confused state of growth substance terminology throughout the literature is reflected in the arbitrary classification recently adopted by the International Phytohormone Conference in Paris (29, p. 48). According to this preliminary report, substances inducing analogous activity are divided into two groups, on the basis of their assumed presence in the "vegetable cell" and their assumed absence "in the plant."

The term "vegetable cell" (29, footnote p. 48) may properly refer to any member of the plant kingdom, including the fungi. Regardless of whether or not the term "vegetable cell" is meant to apply only to the higher plants, the assumption that auxin A, auxin B, and auxin-A-lactone, but not indoleacetic acid, are present "in the plant" is incorrect. Indoleacetic acid has been isolated from fungi, but not from green tissue. The three auxins just mentioned have not been isolated from the green tissue of the higher plants as has been distinctly implied in the outlined nomenclature and throughout the literature.

Since the literature has been concerned primarily with the action of growth substances in higher plants, and the principal value of these findings lies in their possible application to green tissue, there should be no necessity for masking the fact that a crystalline growth substance of known composition and structure has not yet been isolated from the green tissue of the higher plants. It has likewise not been shown that growth substances such as indoleacetic, indolebutyric, and naphthaleneacetic acids are not present in the green tissue of higher plants. While it may be a matter of speculative interest as to the probability of the presence or absence of certain growth substances in plants, the terminology and nomenclature of growth substances and the explanation of their action should be concerned first of all with the facts at hand.

With respect to the qualifications of a plant hormone, Kögl (54, p. 53) remarks that all substances of an analogous activity cannot be considered as hormones either in plants or animals—that the term hormone must be limited to "biological catalysts of organic nature which are used by the organism itself to bring about the various physiological effects." It is a matter of interest that ethylene is the only substance of known composition and structure which meets this specification for the higher plants. Auxin A cannot meet this specification since it has not been isolated from the green tissue of higher plants, and is not known with certainty to occur in green tissue, notwithstanding the various indirect methods used in an

attempt to demonstrate this as a probable fact. Although the similarity between the physiological activity of ethylene and the crystalline growth substances is very striking (20), sufficient data have not been presented to determine the status of this gas as a plant hormone. Particularly are results such as those reported by van der Laan (58) and Michener (69) inadequate for determining the status of ethylene, since no consideration was given to. the well-known influence of different concentrations on the type and degree of characteristic growth responses induced in different plants. Thimann (98, p. 558) remarks that if "ethylene reduces production of the growth hormone" as claimed by van der Laan (58) for *Avena* and *Vicia*, "this can scarcely cause root formation" (98, p. 558). Schlenker and Rosenthal (85, p. 49) also indicate that the growth induced (by ethylene) on the upper side of leaves is not associated directly with an increase in growth substance or its distribution. There is much evidence that ethylene is a normal constituent of plant tissue, occurring in various organs of numerous species (21, 22, 23, 71). The relation of ethylene to metabolic activity (20, 81, 127) is in itself a matter of considerable interest. Also, the disappearance of chlorophyll results from treatment with ethylene and crystalline growth substances (20; 32; 37; 42, p. 456). The exclusion of ethylene as a plant hormone merely because it appears not to act exactly like some other growth substance, such as indoleacetic acid, is a view which fails to consider that no two growth substances act alike as pointed out by Nielsen and Sing-Fang (72, p. 377). Naphthaleneacetic and indoleacetic acids do not act alike for growth promotion (124) nor for root formation (43, 124), yet both are accepted growth-promoting and root-forming substances. The fleshy roots induced in gladiolus corms treated with the K-salt of naphthaleneacetic acid were distinctly different in appearance from the normal types of roots in corms treated with the K-salt of indolebutyric acid or with tap water (126). A similar qualitative difference was mentioned by Oliver (73) for roots induced on cuttings of commercial varieties of shrubs treated with naphthaleneacetic acid and indolebutyric acid.

The importance of relations between chemical structure and physiological activity was realized from the start, since a particular physiological response could be brought about by different substances not closely related in structure but not by many of the more closely related substances. It is significant that most of the discussions relating to the structural specificity of growth substances have emphasized the apparently close structural relationships between distantly related substances but say little about those more closely related compounds which show little or no physiological activity. The relative activity of alpha and beta forms of indoleacetic and naphthaleneacetic acids serve as excellent examples of the difficulties involved. In this case, β-indoleacetic and α-naphthaleneacetic acids are the active forms, whereas α-indoleacetic and β-naphthalene-

acetic acids represent the inactive forms. The question of whether the indole nucleus is structurally similar to, or greatly different from, the naphthalene nucleus must be settled mainly on the basis of physiological activity and not according to physical properties.

If it becomes necessary as recently reported by Koepfli, Thimann, and Went (55) to use a particular type of physiological response, such as cell elongation, exhibited in one test object (*Pisum*) as the criterion for specificity, and then exclude or include certain substances according to their action on another test object (*Avena*), it means that the relationships are not fundamental for plant tissue but only for a particular species under the special conditions of the test. Their choice of *Pisum* as the basic test object is of interest because previously absolute reliance had always been placed on the *Avena* test. However, in *Avena* a number of growth substances (e.g. indolepropionic and coumaryl-acetic acids) are inactive (98, p. 551; 99) and the active substances do not exhibit a stoichiometric relationship such as Went and Thimann claim for the pea test (116, p. 135). Activity in green tissue indicates no such relationship, since at the minimum active concentrations we have found great differences in the molar equivalents of different substances.

Without discounting the value of the *Avena* and *Pisum* tests, it still remains to be demonstrated that the action of growth substances in these test objects is representative of that in green tissue of the higher plants. Particularly is it evident from tests similar to those reported by Skoog (89) that the curvature values obtained with *Avena* for substances extracted from other plants are not reliable indications of the action of these substances in green tissue. Schmitz (86) and Meyer (68) could find no close correlation between growth response and the amount of extractable growth substances. The mistake which Skoog made of assuming that the transport in *Avena* is a measure of transport in the tomato and squash leads to considerable confusion in the literature. Considering this and other limitations of the *Avena* test, Kögl's (54, p. 53) discussion of structural specificity must be considered from the standpoint of the specificity of the "seedling tip test" rather than the specificity of growth promotion or cell elongation for plants in general. His remark (54, p. 53) that root formation is "less dependent on specific structure than the seedling tip test" is of particular interest since the same substances he mentions as being only "distantly related to β-indolylacetic acid" are considered by Koepfli et al. (55) as having similar structural characteristics for growth promotion in *Pisum*. That any close structural relationship should exist between indoleacetic acid and other substances active for root formation was not to be expected when it has been known for several years that naphthaleneacetic and indolebutyric acids are much more effective in green tissue than any other substances (127).

The question of the relation between chemical structure and physiological activity has been concerned mainly with a study of substances active on a few etiolated test objects rather than with plants in general and green tissue in particular. Growth in intact green plants has never been shown to result from the application of simple acids as has been claimed for growth in test objects such as *Avena*, *Pisum*, *Helianthus*, etc. The status of the "acid growth" effect is far from settled since its validity depends upon the unproved assumption that the pH of the cell contents in intact green plants is appreciably lowered. It must be remembered that under the conditions in which intact green tissue plants have been treated, as in our laboratory, there is afforded no opportunity for immersing or bathing the tissue in acid buffers so that all regions exhibiting a growth response would be in equilibrium with the applied preparations. Even in the use of small isolated segments, a few millimeters in length, van Santen (82) has emphasized the difficulty of obtaining an equilibrium between the external acid solution and the cell contents, which has probably not been attained by all other workers. If simple acids can promote cell elongation in *Avena* without lowering the pH of the cell contents, then simple acids must be considered as active for cell elongation in *Avena*, which would mean cell elongation was not a highly specific response.

The stimulus concept, particularly as interpreted by Fitting (31), has been opposed from the start by proponents of the specificity concept (Utrecht school) for the action of growth substances. Considering the relatively large number of growth substances which induce similar responses in plants, Jost (50, p. 748) remarked that these substances appear to act as chemical stimulants in the sense suggested by Fitting. Boysen Jensen (11, p. 525) also expresses a similar opinion. Went (112, p. 452; 114, p. 55) and Went and Thimann (116, p. 244–247) are opposed to the stimulus concept. Their arguments in favor of the specificity concept are based largely on claims relating to the "stoichiometric reaction," the "master-reaction," and the manner in which "auxin" is assumed to regulate the transport of other growth substances or is assumed to activate other factors (114, p. 55). These assumptions not only lack substantiation for all test objects, as pointed out in this section and in other sections, but substantial data opposed to these claims continue to accumulate. There appears to be no specificity of action for any of the substances classed as plant hormones or growth substances which can compare with the specificity of action for animal hormones.

Age and relative activity of cuttings. All leaves on the tomato plant do not have the same capacity to root. Leaves from the upper part of the plant produce more roots than those from the lower part. An example of this is shown for duplicate sets of leaves in Table I and for three upper leaves in Figure 3 A. In Table I, and in other tables with similar data, the

FIGURE 3. Relation between concentration, number of roots, and length of tissue from which roots emerged. (A) Root formation (seventh day) in tomato leaves of different age treated for 24 hours with solutions of indolebutyric acid. Youngest leaf on left and oldest on right, in each set. (Leaflets trimmed when photographed.) (B) *Chrysanthemum* (var. Red Bird) 13 days after treatment with indolebutyric (top row), naphthaleneacetic (middle row), and indoleacetic (lower row) acids.

results are arranged so that a quick evaluation of the data may be accomplished merely by referring to column totals and group totals which reflect all important trends appearing in the main body of the table. Thus, in Table I the relation between concentration of indolebutyric acid and the number of roots induced by the treatment is readily seen in the column

TABLE I

Effect of Age of Leaf on Root Formation in Tomato Petioles.
Average Number of Roots per Cutting

Order of leaves on plant from top to bottom	Indolebutyric acid, mg. per liter								Group totals
	Water		0.032		0.10		0.32		
1	0	0	0	0	0	0	10	45	55
2	0	0	0	4	7	11	39	36	97
3	0	0	0	2	10	5	32	28	77
4	0	0	0	0	0	0	9	10	19
5	0	0	0	0	0	0	2	1	3
Column totals	0	0	0	6	17	16	92	120	

totals. The total performance (concentration effect) of the five leaves of different age (column totals) is represented by the totals for each series of values and shows that with increasing concentrations of growth substance, the number of roots in the tomato leaf cuttings increases. Similarly, the relation between the age of leaf and the number of roots induced by treatment with indolebutyric acid is shown by group totals. The total performance of leaves, according to their age is represented by the totals (last column) for all four concentrations for each of the five age series. While it does not fall within the scope of the present paper to study the deviations in the many separate tests, all data essential for such a study appear in the main body of the table.

Relative activity is also of importance. Leaves on slow-growing plants were not so sensitive as leaves located at a similar position on plants showing a normal vigorous growth. Bonny Best and Marglobe varieties of tomato do not grow at the same rate in the vegetative stage; hence some of the differences obtained in the root formation tests may be accounted for by differences in the rate of growth of the two varieties of the same age. However, either variety may be used with about equal success in the root formation tests.

The rooting response of the tomato leaf according to the age and relative activity of the cutting is representative of stem cuttings of most plants. Generally, the most striking examples are to be found in plants which are most difficult to root from cuttings. This applies irrespective of whether the cuttings have been treated with growth substance preparations. As contrasting examples leafless cuttings of the commercial fruiting apple (Rhode Island Greening) and *Hibiscus* may be cited (Fig. 2 A and Table II). Tip cuttings of the apple (varieties Grimes Golden and Rhode Island Greening) taken from November to February rooted consistently (75 to 100 per cent) in five weeks when treated with indolebutyric acid (20 to 60 mg. per liter for 24 hours), whereas cuttings made from the remainder of the material on the same shoot showed less rooting or no root-

ing. A four-hour treatment with higher concentrations was also effective (Fig. 2 A). Control cuttings did not root in this time. The special method of burying treated cuttings in moist peat moss was described previously

TABLE II

RELATIVE ACTIVITY OF ACIDS AND SALTS FOR ROOT FORMATION IN HIBISCUS CUTTINGS OF DIFFERENT AGE. AVERAGE NUMBER OF ROOTS PER CUTTING

Mg. per liter	Acids			Group totals	K-salts			Group totals	Class totals*
	Indole-acetic	Indole-butyric	Naphtha-leneacetic		Indole-acetate	Indole-butyrate	Naphtha-leneacetate		
Part A—Tip cuttings									
80	3	51	4	58	4	14	3	21	79
40	3	10	2	15	12	13	8	31	46
20	0	7	7	14	6	38	5	49	63
10	2	5	1	8	1	7	4	12	20
Water	7	1	0	8	0	1	2	3	11
Column totals	15	74	14	103	23	73	22	116	
Part B—Middle cuttings									
80	14	122	4	140	5	134	1	140	280
40	9	130	17	156	1	189	25	215	371
20	10	111	6	127	12	120	3	135	262
10	0	67	3	70	11	11	6	28	98
Water	0	2	0	2	0	0	5	5	7
Column totals	33	431	30	495	29	454	40	523	
Part C—Basal cuttings									
80	8	162	82	252	6	144	22	172	424
40	13	206	42	261	4	192	16	212	473
20	6	103	11	120	9	62	7	78	198
10	1	57	4	62	5	42	1	48	110
Water	3	1	0	4	0	4	1	5	9
Column totals	31	529	139	699	24	444	47	515	
Totals (Part A, B, C)	79	1034	183	1297	76	971	109	1154	

* Summation of values for all acids and all salts.

(39, p. 240). On the other hand, leafless tip cuttings of *Hibiscus syriacus* given the same treatment rooted poorly as compared to cuttings made from the older material (Table II). Leafy cuttings of many other kinds of plants showed a similar critical difference according to the type of material used for the cuttings. Succulent tips of *Chrysanthemum* shoots were

much more sensitive than older portions below. Tip cuttings of *Lonicera maacki* treated with indolebutyric acid (20 mg. per liter) rooted poorly when taken in July and best when taken in September, as compared with the basal portions of the shoot. Young shoots taken the latter part of April rooted in 16 days when treated with concentrations of 5 to 10 mg. per liter. The bases of *Arctostaphylos Uva-ursi* shoots rooted better than the tip portions when the cuttings were treated with indolebutyric acid (5 to 10 mg. per liter) during October. Tip cuttings of *Callicarpa japonica* var. Koreana responded to lower concentrations than basal cuttings when taken the latter part of June. During June the tip cuttings of *Kolkwitzia amabilis* treated with indolebutyric acid (20 mg. per liter) were well rooted before any roots had emerged from the basal cuttings. Thus in root formation tests with green tissue the results are noticeably influenced by the condition of the tissue used for cuttings. Others have reported similar effects (49, 67).

TABLE III

INFLUENCE OF AGE OF TISSUE AT BASE OF PETIOLE ON ROOT FORMATION
IN TOMATO LEAVES TREATED WITH INDOLEBUTYRIC ACID

Position of basal cut	Tap water preparations				Distilled water preparations				Class totals*
	Concn. mg. per liter			Group totals	Concn. mg. per liter			Group totals	
	Water	0.032	0.32		Water	0.032	0.32		
At base	0	2	81	83	0	11	110	121	204
12 mm. above	1	27	32	60	0	13	10	23	83
25 mm. above	0	4	44	48	0	1	10	11	59
Column totals	1	33	157	191	0	25	130	155	

* Summation of values for tap water and distilled water.

Age of tissue at base of cutting. Propagators are familiar with the fact that at any particular time of the year the age of the tissue at the base of the cutting is an important limiting factor in root formation. Several reports have discussed this question (10, 40, 41, 118, 119, 122). Results for tomato leaf cuttings taken during the summer show that removing the basal portion before treatment reduces the number of roots formed (Table III). The reverse is true for tests carried out during the winter (Table IV). Similar seasonal variations are known for other test objects. Van Overbeek and Went have given this information for the pea (76) and Söding for *Cephalaria* (96). For *Cephalaria* the difference is much greater than is the case for either the pea or the tomato.

Effect of leaf area. Root formation in tomato leaf cuttings was approximately proportional to the amount of leaf area (Table V). The American holly (*Ilex opaca*) responds in a similar manner (122). Internodal cuttings of the Concord grape root with difficulty or not at all, even though it may

be shown that there is an abundance of stored food materials (121). Treatment of such cuttings with 20 to 80 mg. per liter of indolebutyric acid induced the formation of many roots, particularly in the case of the higher

TABLE IV

RETREATMENT OF TOMATO LEAF CUTTINGS WITH INDOLEBUTYRIC ACID (1 MG. PER LITER)

Time basal portion was removed	Average number roots per cutting			Length of petiole (mm.) from which roots emerged*		
	Length cut off			Length cut off		
	None	1 cm.	2 cm.	None	1 cm.	2 cm.
After 24-hour treatment with growth substance	28	27	11	40	43	35
After 24-hour treatment with water (control)	0	0	2	0	0	0
After 24-hour treatment with growth substance then retreated	49	36	19	50	58	30
Before treatment with growth substance	39	90	72	35	53	35
Before treatment with water	1	8	0	0	18	0

* Measured from basal cut upward.

TABLE V

EFFECT OF RELATIVE LEAF AREA ON ROOT FORMATION IN TOMATO PETIOLES TREATED WITH INDOLEBUTYRIC ACID

No. (or part) of leaflets on cutting	Tap water preparations				Distilled water preparations				Class totals*
	Concn. mg. per liter			Group totals	Concn. mg. per liter			Group totals	
	Water	0.032	0.32		Water	0.032	0.32		
All 5	1	122	125	248	2	26	119	147	395
1/2 of all 5	2	18	73	91	1	41	43	85	176
1/2 lower pair	0	0	11	11	0	0	13	13	24
Terminal	0	0	3	3	0	1	0	1	4
None	0	0	0	0	0	0	0	0	0
Column totals	3	140	212	353	3	68	175	246	

* Summation of values for tap water and distilled water.

concentrations. Thus neither buds, leaves, nor nodal tissue were essential for root formation. However, in addition to lacking the capacity to form adventitious buds, the internodal cuttings failed to develop a normal type of branched root system. All of these cuttings died eventually after rooting, since adventitious shoots were not formed.

Van der Lek (66) demonstrated the importance of buds and leaves for root formation in cuttings. Having recognized the hormonal nature of root-forming substances furnished by buds and leaves, van der Lek laid the fundamental groundwork for our present knowledge of root formation. Similar results for the bud and leaf effect were reported by the present authors for currant (*Ribes*), *Prunus tomentosa*, grape, *Viburnum*, *Hibiscus*, etc. (121). Bouillene and Went (10) later furnished much additional information concerning this effect and formulated a number of postulations for the action of the special substances involved. The influence of buds and leaves is both a quantitative and qualitative one, since the formation of branch roots, number of roots, and relative diameter of roots were involved (121). The influence of leaf area on root formation in cuttings of commercially important plants has been mentioned in other reports (43, 49). Response and recovery of plants following treatment with growth substance were also influenced by the relative amount of leaf area (42).

TABLE VI

ROOT FORMATION IN TOMATO LEAVES TREATED WITH ALCOHOLIC
SOLUTIONS OF INDOLEBUTYRIC ACID

Indolebutyric acid, mg./l.	Percentage of ethyl alcohol					Group totals
	Water	0.01	0.032	0.1	0.32	
1.0	85	135	88	7	0	315
0.1	18	36	17	0	0	71
0.01	32	10	0	0	0	42
Water	7	0	0	0	0	7
Column totals	142	181	105	7	0	

Alcoholic solvents. In confirming the results of Schlenker and Mittmann (84) and Pearse (77) relating to the application of growth substances directly to the leaves of intact plants, it was noted that preparations containing from 0.1 to 1.0 per cent ethyl alcohol were more effective when sprayed on leaves of the tomato than similar preparations minus alcohol. A similar alcohol effect was observed for root formation in the tomato leaf. Results in Table VI show that preparations containing 0.01 per cent ethyl alcohol were more effective than similar preparations not containing alcohol but which contained indolebutyric acid in concentrations of 0.1 to 1.0 mg. per liter. The alcohol itself was not active for root formation and it was definitely toxic in concentrations of 0.1 per cent or higher. Methyl and iso-propyl alcohols gave similar results, except that these two alcohols proved less toxic. The optimal effect of alcohol occurred between the values 0.0032 to 0.032 per cent. It appeared in some cases that growth substance preparations containing from 0.25 to 1.0 per cent ethyl alcohol were more effective on woody cuttings than similar preparations minus alcohol. These

results indicate that the penetration of growth substance may be increased by the presence of small amounts of alcohol.

The influence of the solvent and the penetrating characteristics of the growth substance have probably not been given sufficient consideration in growth substance studies. Particularly from the standpoint of practical application, the question of solvents may prove of considerable importance. The use of growth substance for several different purposes such as delaying the growth of buds or otherwise regulating the rate of growth, is now limited because of the difficulty with which lanolin and water preparations penetrate relatively impervious outer tissue when used in nontoxic concentrations. However, in some recent tests in which the growth of flower and leaf buds on fruit trees was delayed by sprays containing naphthaleneacetic acid, it was found that water solutions as well as preparations containing "Penetrol" (an oxidized sulfanated petroleum product used as a carrier for insecticides) were effective. These results indicate that when a sufficiently high concentration of naphthaleneacetic acid was used penetration was obtained, but "Penetrol" was a more effective carrier than water. Even in the case of soft tissue, certain oils were found to be much better carriers of the growth substance than lanolin (38). It seems likely, also, that some of the so-called acid and pH effects may involve penetration as suggested by Albaum, Kaiser, and Nestler (2). In the case of *Nitella*, penetration of indoleacetic acid was most rapid at the lower pH values, but the pH of the cell contents was not affected even by preparations buffered at pH 4.1. The results reported by these authors do not lend support to the suggestion by J. Bonner (8) and Went and Thimann (116, p. 130) that the acidity of the applied growth substance lowers the pH of the tissue.

Retreatment of cuttings. In these tests a portion of the basal part of the cutting was removed after an initial 24-hour treatment with growth substance in order to determine the extent of the upward movement of the basally applied indolebutyric acid and also to determine to what extent the natural occurring substances, presumed to be essential for root formation, had moved toward or accumulated at the base of the cutting. Some of the cuttings treated in this manner were subjected to a second 24-hour treatment with indolebutyric acid and some were transferred to water to serve as controls. In the case of the tomato leaf cuttings, a similar portion was removed from the basal end before the initial 24-hour treatment with indolebutyric acid in order to determine the effect of the age of tissue at the base on root formation. Results for the tomato leaf are shown in Table IV. The removal of a basal segment 1 cm. in length failed to reduce the number of roots formed or the length of tissue along the petiole from which the roots emerged. In contrast, the removal of a 2-cm. segment reduced the number of roots to less than one-half and the length of the rooting region

to seven-eighths. Thus the effect of the initial 24-hour treatment with growth substance was not eliminated by cutting off the basal 2-cm. segment, although some reduction occurred in the number of roots formed. Similar cuttings retreated for a second 24-hour period formed more roots, which is an indication of an additive effect. In this case, also, there was better rooting on retreated cuttings from which a 1-cm. segment had been removed as compared to the removal of a 2-cm. segment.

Results with tomato leaf cuttings are interpreted as meaning that the basally applied indolebutyric acid was absorbed and transported upward for a distance of more than 2 cm. and in an amount sufficient to induce root formation. This conclusion is opposed to the assumption (17; 18; 76; 114; 116, p. 200–202) that there is no appreciable upward movement of basally applied growth substance and hence any root formation resulting from basal application is to be explained on the basis that there is an accumulation by downward movement of all of the substance or substances in the cutting which are necessary for root formation. According to this assumption, cutting off the treated base would remove all or most of the substances essential for root formation which are presumed to have accumulated in the basal region of the cutting. Our results do not support such an assumption. Cooper's data (17, Exp. C) do not support his conclusion that cutting off the basal three-fourths of an inch of treated leafy lemon cuttings nearly eliminated the effect of the treatment. The 4.9 roots represent 39 per cent of the number (12.6) obtained on similar cuttings from which the bases were not removed after treatment. Similar results were reported at a later date (18). Our interpretation of his results is that the basally applied indoleacetic acid which he used moved upward in the lemon cuttings for a distance of more than three-fourths of an inch since the removal of this length did not eliminate, although it reduced, the effect of the treatment as implied. The failure to obtain more roots with a second treatment (retreatment) does not agree with our results with tomato leaf cuttings or with cuttings of Concord grape, *Euonymus radicans*, *Hibiscus syriacus*, *Ilex opaca*, *Rosa* var. Excelsa, and *Viburnum opulus*. Leafless cuttings of grape and *Hibiscus* were used, but cuttings of the other four species had leaves. In contrast to the tomato leaf, in which cutting off the base did not eliminate the effect of the treatment, the effect of the treatment on grape cuttings was eliminated by cutting off the base. Thus the leafless grape cuttings responded like Cooper's *leafless* lemon cuttings with respect to the elimination of the effect of the initial 24-hour treatment, but unlike the lemon, the grape cuttings responded to retreatment. Attention is again called to the fact that the effect of the treatment was not eliminated by cutting off the bases of *leafy* lemon cutting used by Cooper, although his *leafless* cuttings exhibited such an effect.

Hibiscus cuttings differed slightly in their response from that reported

for grape. Not only did retreatment of *Hibiscus* cuttings cause an increase in the number of roots formed, but in the lot from which one and one-half inches had been cut off, root formation was better than in a similarly treated lot from which the bases had not been removed. The corresponding lot which had not been retreated showed less reduction in root formation than similarly treated lots from which one-half and one inch, respectively, had been removed. According to the "local mobilization" effect postulated by Cooper (17) and Went and Thimann (116), it would be expected that the greater the length of the segment cut off after the initial treatment, the greater the chance that the cuttings would be depleted of all of the substances essential for root formation (accumulated at the base) and hence would result in a much greater reduction in root formation. The assumption would not hold for the grape or the *Hibiscus*.

Retreatment of leafy cuttings of *Euonymus*, *Ilex*, *Rosa*, and *Viburnum* from which three-fourths of an inch had been cut from the basal end, showed better rooting than similar cuttings which were not retreated. Results for *Ilex opaca* are shown in Figure 4 A. It is to be noted that indoleacetic acid was not active for root formation under conditions in which indolebutyric acid was highly active (Fig. 4 A). Additional tests with *Ilex opaca* showed that three such segments could be cut off successively after each of three 24-hour treatments with indolebutyric acid without depleting the cuttings of substances essential for root formation. Thus at the end of a 72-hour period a total of two and one-fourth inches had been cut from the basal ends of the *Ilex* cuttings. Our interpretation is that cutting off a portion of the base of *Ilex* cuttings did not deplete it of all the substances essential for root formation, even though some reduction may have occurred.

Results with retreated intact tomato plants indicated, also, that failure to respond as originally, was not associated with the loss of active substances (125, p. 308). In these tests no portion of the plant was removed as was the case with woody cuttings. Thus, the initial application of growth substance, or the increase due to redistribution (and possibly to the formation of more growth substance) in geotropically or phototropically stimulated organs, alters the tissue so that continued treatment or retreatment does not have the same effect. This possibility was not considered by Cooper (17, 18) or by Went and Thimann (116) in connection with their assumption that all substances essential for root formation are removed when the lower three-fourths of an inch of woody cuttings is cut off within 24 hours after basal treatment with growth substance solutions. In our tests retreated control cuttings (bases not cut off) did not show an increase in the number of roots proportional to the increase in duration of treatment from 24 to 48 hours. This is a further indication that the tissue is appreciably altered during the first 24 hours of treatment. Emphasis
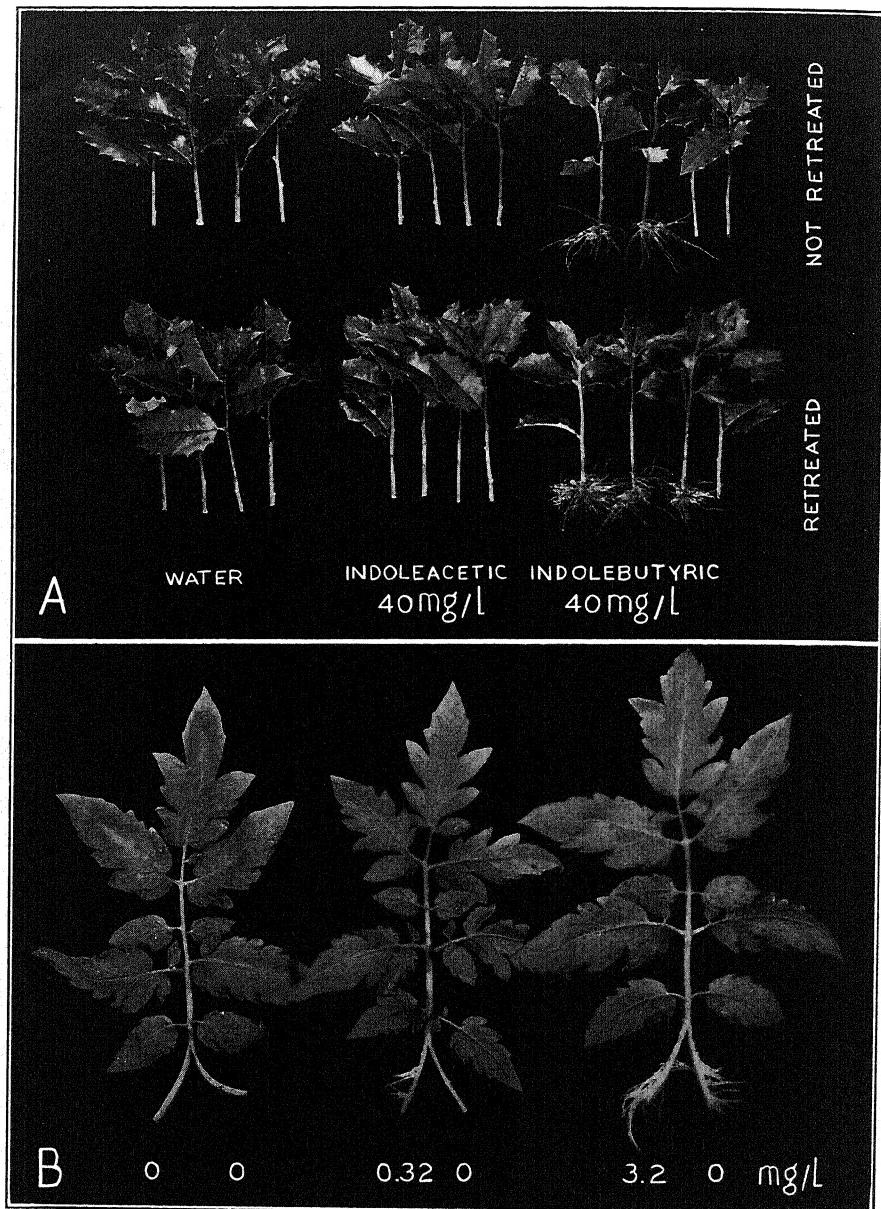
FIGURE 4. (A) *Ilex opaca* cuttings 39 days after treatment with growth substance solutions. Top row treated 24 hours and then bases cut off; lower row treated 24 hours, then basal three-fourths inch cut off and treated for an additional 24 hours. (B) Tomato leaves slit at base and left half placed in indolebutyric acid and right half in tap water (in separate vials). Photographed 9 days later.

should be placed upon the fact that results obtained with cuttings of seven different genera show distinct differences in response to retreatment.

Hellinga (36, p. 11) has pointed out that Cooper's postulation of rhizocaline in cuttings is an unnecessary prerequisite for root formation, since in her tests retreated cuttings from which the treated ends were cut off showed increased root formation when placed in sugar solutions. We obtained a similar increase in root formation, in our tests, without the use of sugar or other supplementary food factors. Pearse (78) and Dorfmüller (25, p. 483) likewise were unable to verify Cooper's results relating to retreatment of cuttings. Since the "local mobilization" hypothesis has not been substantiated, it would appear to have no general application.

Retreatment tests such as the ones described are complicated by the fact that in many varieties the position of the basal cut is of considerable importance in determining the number of roots formed. In the case of tomato leaf cuttings taken during the winter, removal of a portion of the base before treatment resulted in the production of more roots (Table IV). This militates against the idea that there is a limited amount of substances essential for root formation so that reducing the length of the cuttings as postulated by Went for the pea hypocotyl (114) should reduce proportionally the number of roots formed. Such an assumption has no application to short cuttings which root as readily as or more readily than longer cuttings. However, in retreatment tests the influence of the position of the basal cut should be accounted for satisfactorily. In addition to the increase in the number of roots formed, retreatment of tomato cuttings caused root formation in tissue above the basal cut (Table IV). Since our results show that there is some additive effect of retreatment (increased number of roots), it serves as evidence that the transport of applied growth substance extended upward beyond the three-fourths of an inch postulated by Cooper (17). This is discussed further in the section dealing with transport.

Deferred treatment of *Ilex opaca* cuttings with an interval of 76 days between the time the cuttings were made and treated with growth substance was reported as effective by Stuart and Marth (97). Cooper and Went (19) obtained similar results for Hamlin sweet orange, *Camellia*, and *Papaya*. We obtained similar results with *Camellia japonica* var. Alba Plena, which is one of the most difficult varieties to root, and also with *Taxus cuspidata* and *Buxus*. The interval in our tests was from six weeks to five months. This type of deferred treatment was effective regardless of whether the cuttings had been treated previously with indolebutyric acid. There was thus no indication of a pronounced additive effect in these tests such as was obtained in tests where retreatment was administered 24 hours after the initial treatment. Cooper and Went (19) failed to distinguish between deferred treatment and retreatment. It is thus not known whether, in their tests, the slight increase in number of roots was due to an

additive effect of the initial treatment and that given three weeks later, or whether it was due solely to the effect of the second treatment. Whereas Cooper and Went (19) made no mention of control cuttings (those treated initially with water and after three weeks treated with growth substance), we found that both control and treated cuttings responded essentially the same to treatment with growth substance at a later period. It would appear that the changes which occur in some cuttings (*Camellia, Taxus,* etc.) while remaining in the rooting medium during a period of several weeks or several months, are comparable to changes occurring in shoots on the intact plant in which case the condition of the tissue and time of year cuttings are taken determine their capacity to form roots. In the case of *Taxus,* the callus knobs were removed from some cuttings just prior to treatment with indolebutyric acid. In these particular cuttings more roots were formed eventually and they emerged from a greater length of stem tissue as compared with those which did not have the callus knob cut off. However, the treated *Taxus* cuttings with intact callus knobs rooted in a shorter time than those from which the callus knobs had been removed. From the practical standpoint it is advisable to treat cuttings with intact callus. In cases where root formation seldom occurs in the absence of callus, as in *Camellia, Rhododendron,* and *Vaccinium* (blueberry), an initial treatment with growth substance would be expected to hasten callus formation and thereby prove beneficial from the standpoint of providing at an earlier time one of the conditions which favors rooting. Subsequent treatment at a later period (retreatment) might, then, induce root formation in certain species which do not respond readily to the initial treatment. The Alba Plena variety of *Camellia* belongs to this class. In contrast, other varieties of *Camellia* (Dixie, Sarah Frost, and var. Rosea Grandiflora) respond so readily to an initial treatment with indolebutyric acid that a deferred treatment or a retreatment would be unnecessary. Results with *Taxus* cuttings indicated that callus knobs retarded the absorption and upward movement of the basally applied indolebutyric acid, so that root formation in this type of treated cutting was mainly basal. These results agree with those reported by Stuart and Marth for *Ilex opaca* (97).

Relationships associated with concentration. Although long periods of treatment resulted in the formation of more roots than occurred with short periods, there was no proportional relationship between the concentration of growth substance and the duration of treatment (Table VII). Likewise, there was no close relation between the amount of solution absorbed by cuttings and the number of roots induced (Tables VII and VIII). On the basis of the amount of growth substance calculated to be present in the volume of solution absorbed by cuttings, the total amount of growth substance was of less importance for rooting than the concentration applied. However, where extreme differences in conditions influencing transpira-

tion were provided, differences in root formation resulted (Tables VII, VIII, and IX). Thus atmospheric conditions (quantity and quality of light, temperature, and humidity) are important factors in determining the relative effectiveness of the treatment regardless of the concentration of growth substance used. These results are in agreement with those reported by the writers (42) and van der Lek and Krijthe (67) for the absorption and transport of growth substances.

TABLE VII

RELATION BETWEEN CONCENTRATION OF GROWTH SUBSTANCE, DURATION OF TREATMENT, AMOUNT OF SOLUTION ABSORBED, AND ROOT FORMATION IN CUTTINGS OF PACHYSANDRA TERMINALIS TAKEN IN OCTOBER. AVERAGE VALUES PER CUTTING

Conc. indolebutyric acid, mg./l.	4 hours		8 hours		16 hours		32 hours		64 hours		Class totals*	
	Soln. absorbed in cc.**	No. of roots	Soln. absorbed in cc.**	No. of roots	Soln. absorbed in cc.**	No. of roots	Soln. absorbed in cc.**	No. of roots	Soln. absorbed in cc.**	No. of roots	Water loss	No. of roots
A. Cuttings exposed during treatment												
160	1.5	26	3.0	21	5.5	26	10.2	27	17.5	0	38	100
80	0.6	29	3.1	64	5.0	39	13.0	66	14.6	111	36	309
40	1.0	35	2.9	43	4.5	37	8.0	77	14.7	34	31	226
20	1.0	28	1.7	13	3.5	22	7.1	53	14.6	17	28	133
10	1.7	35	1.9	8	4.0	16	9.0	19	16.9	29	34	107
0	1.8	21	1.9	8	5.0	8	10.4	12	14.4	24	34	73
Column totals	7.6	174	14.5	157	27.5	148	57.7	254	92.7	215	201	948
B. Cuttings under bell jar during treatment†												
160	0.2	22	0.5	29	1.0	12	1.7	3	3.0	110	6	176
80	0.3	13	0.2	29	1.3	9	1.8	29	3.9	40	8	120
40	0.2	48	0.4	10	1.0	12	1.9	44	2.8	21	6	135
20	0.2	31	0.2	14	1.3	11	2.1	43	3.0	21	8	120
10	0.2	12	0.2	25	1.1	5	1.1	37	2.0	9	5	88
0	0.3	17	0.3	18	1.1	6	1.1	16	2.0	24	5	81
Column totals	1.4	143	1.8	125	6.8	55	9.7	172	16.7	225	38	720

* Summation of values for all five time periods.
** Represents difference in volume before and after treatment.
† Exit hole at top of bell jar left open.

Considering the relatively large number of substances which have been reported as active for root formation, it is a matter of interest to know how one may distinguish between substances which are active and those which are not. Treatment of cuttings of more than 100 species with growth substances indicated that no sharp line of distinction exists. Most species were consistently more sensitive to indolebutyric acid than to either indoleacetic or naphthaleneacetic acids. Some plants were about equally sensi-

TABLE VIII

RELATION BETWEEN ABSORPTION OF GROWTH SUBSTANCE SOLUTION UNDER DIFFERENT
ATMOSPHERIC CONDITIONS AND ROOT FORMATION IN CUTTINGS OF CHRYSANTHEMUM
VARIETY RED BIRD. TREATMENT FOR 24 HOURS.
ROOT COUNTS END OF TENTH DAY

Treatment	Dura-tion of treat-ment, hrs.	Dark room (10° C.)		Laboratory (18°–31° C.)		Greenhouse, glass painted (18°–28° C.)		Greenhouse, clear glass (27°–38° C.)	
		Soln. lost, cc.	Av. No. roots per cutting	Soln. lost, cc.	Av. No. roots per cutting	Soln. lost, cc.	Av. No. roots per cutting	Soln. lost, cc.	Av. No. roots per cutting
A. Cuttings exposed during treatment									
Controls (tap water)	1	1.5	14	1.6	13	0.5	11	3.0	4
	4	3.0	11	4.5	13	3.5	16	5.5	11
	16	7.5	12	2.5	12	5.5	11	6.5	13
Column totals		12.0	37	8.6	38	9.5	38	15.0	28
Indolebutyric acid, 1.25 mg. per liter	1	1.5	13	1.5	13	1.0	8	2.5	14
	4	2.5	13	4.5	17	5.0	26	7.0	25
	16	7.5	27	3.0	40	5.5	29	6.0	42
Column totals		11.5	53	9.0	70	11.5	63	15.5	81
B. Cuttings under bell jar during treatment*									
Controls (tap water)	1	1.5	13	0.4	15	0.5	11	1.0	13
	4	3.0	10	3.5	17	5.0	15	5.5	10
	16	3.0	14	2.5	8	2.0	12	4.5	6
Column totals		7.5	37	6.0	40	7.5	38	11.0	29
Indolebutyric acid, 1.25 mg. per liter	1	0.5	13	1.0	21	1.0	13	2.0	12
	4	4.0	23	3.5	17	1.0	17	7.0	46
	16	3.0	18	3.0	31	3.0	18	5.0	33
Column totals		7.5	54	7.5	69	5.0	48	14.0	91

* Exit hole at top of bell jar left open.

TABLE IX

ROOT FORMATION IN TOMATO LEAF CUTTINGS KEPT UNDER DIFFERENT TYPES OF GLASS

House No.	Color or type of glass	Indolebutyric acid, mg. per liter				Group totals
		Water	0.032	0.10	0.32	
1	Clear	0	0	0	0	0
2	Opaque quartz	0	0	0	47	47
3	Blue	4	7	48	31	90
4	Yellow	2	0	72	35	107
5	Red	0	16	46	153	215
Column totals		6	23	166	266	

tive to indolebutyric and naphthaleneacetic acids, and others responded best to naphthaleneacetic acid. Indoleacetic acid was not as effective as indolebutyric or naphthaleneacetic acids. Increasing the concentration of any one of these three substances caused a marked increase in the number of roots induced. In contrast, results with phenyl compounds (phenylacetic acid, phenylpropionic acid, sodium cinnamate, nitrocinnamic acid, aminophenylacetic acid, etc.) have not shown a marked increase in number of roots induced in tomato leaf cuttings, and the effective range was much narrower. The phenyl compounds mentioned are definitely of low or questionable activity for root formation (depending upon the test plant) but as compared with the highly active indole and naphthalene compounds the difference is a quantitative one. The high activity of indolebutyric acid for root formation in green tissue which has been found in all tests reported from this laboratory has also been confirmed by other investigators (13, 78, 79, 88, 102, 103). An attempt to explain growth substance activity solely in terms of results obtained with indoleacetic acid would be highly speculative with relation to root formation.

Besides inducing the formation of a greater number of roots, the highly active indole and naphthalene compounds caused roots to emerge from a greater length of stem or petiole tissue than was normally the case in control cuttings. This relationship was a function of concentration (Figs. 1 C, 1 D, 2 B, 3 A, 3 B, and Tables IV, X, XI). On the basis of this relationship substances may be classed as showing high activity or little or no activity for root formation, since it is a question of inducing root formation in many varieties of cuttings in tissue where roots are not normally formed (49, 102). This applies particularly to cuttings which normally form roots only at the base or only at the nodes. Traub (104) did not refer to this type of response. Growth promotion in the tomato petiole (Table XII) was also a function of concentration and represents a test which is sensitive to concentrations of 1 mg. per liter or less.

In contrast to the results described by Went for the pea hypocotyl

TABLE X

ROOT FORMATION AFTER EIGHT DAYS IN YOUNG SHOOTS* OF CHRYSANTHEMUM (VAR. RED BIRD) TREATED WITH INDOLEBUTYRIC ACID FOR 24 HOURS

Concn., mg./l.	Av. No. roots per cutting	Distance above base (mm.) roots emerged
1.00	34	17
0.32	32	27
0.10	13	5
0.03	4	8
Water	6	13

* Tip cuttings made with basal cut through soft tissue.

TABLE XI

UPWARD MOVEMENT OF ACIDS AND ESTERS AS INDICATED BY ROOT FORMATION ABOVE THE
BASE. DISTANCE IN MILLIMETERS FROM BASAL CUT SURFACE TO UPPERMOST
ROOT ON TOMATO LEAVES AT END OF SEVENTH DAY*

Form of growth substance	Name**	Bonny Best variety				Marglobe variety				Class totals†
		Water	10^{-6} M	10^{-5} M	Group totals	Water	10^{-6} M	10^{-5} M	Group totals	
Acids	IA	0	0	30	30	0	0	0	0	30
	IB	3	21	57	81	0	0	45	45	126
	NA	0	25	74	99	0	23	49	72	171
Column totals		3	46	161	210	0	23	94	117	327
Methyl esters	MIA	0	37	27	64	0	15	4	19	83
	MIB	0	0	51	51	0	4	47	51	102
	MNA	0	27	37	64	0	10	52	62	126
Column totals		0	64	115	179	0	29	103	132	311
Ethyl esters	EIA	0	7	13	20	0	5	50	55	75
	EIB	4	22	58	84	0	0	54	54	138
	ENA	0	25	48	73	0	0	48	48	121
Column totals		4	54	119	177	0	5	152	157	334

* Root counts for these cuttings appear in Table XVII.
** IA = indoleacetic, IB = indolebutyric, NA = naphthaleneacetic.
† Summation of values for both varieties of tomato.

(114, p. 72), our results with different species of cuttings show that basal
application of growth substances is effective for root formation over a
range in concentration of about 10,000-fold, beginning at 10^{-7} and extend-
ing to about 3×10^{-3}M. Concentrations effective for root formation de-

TABLE XII

GROWTH PROMOTION IN TOMATO LEAF CUTTINGS AS INDICATED BY THE PERCENTAGE
INCREASE IN LENGTH OF MARKED SEGMENTS AT THE BASE OF THE PETIOLE

Position of marked segment (5 mm.)	Indolebutyric acid, mg./l.				Group totals	Indoleacetic acid, mg./l.				Group totals
	Water	0.032	0.10	0.32		Water	0.032	0.10	0.32	
Basal	5	6	13	27	51	1	5	10	15	31
Middle	3	7	8	8	26	3	2	7	10	22
Upper	0	0	7	7	14	1	6	5	6	18
Column totals	8	13	28	42		5	13	22	31	

pended upon the species of plant, the condition of material used for cut-
tings, and the duration of the treatment. We have found, for example, that
concentrations less than 10^{-4}M (1 to 10 mg. per liter) were active for root
formation in more than 100 species of cuttings, so that the critical limit
of 10^{-4} molar must be regarded as a special case for the pea. Concentrations

of 1 to 5 mg. per liter are active for root formation in young shoots of most common deciduous shrubs such as *Forsythia*, *Lonicera*, *Spiraea*, *Hydrangea*, *Weigela*, ornamental *Prunus*, etc. Many varieties of rose require less than 5 mg. per liter to give optimum rooting. For example, when flowering

TABLE XIII

INFLUENCE OF COMPOSITION AND CONCENTRATION OF BUFFER ON ROOT FORMATION IN
TOMATO LEAF CUTTINGS TREATED WITH SOLUTIONS OF INDOLEBUTYRIC ACID
AND ITS POTASSIUM SALTS. AVERAGE NUMBER OF ROOTS PER
CUTTING AT END OF SEVENTH DAY

| Buffer (pH values for 10^{-3} M solns.) | Kind of growth substance | Molar concn., growth sub-stance | Bonny Best variety | | | | Marglobe variety | | | | Totals for each buffer* |
| | | | Molar concn. of buffer | | | | Molar concn. of buffer | | | | |
			Dist. water	10^{-7}	10^{-5}	10^{-3}	Dist. water	10^{-7}	10^{-5}	10^{-3}	
A. Acid buffers pH 4.9–5.2											
Phthalate NaOH pH 4.9	Indole-butyric acid	10^{-6} 10^{-7} Ck.**	5 0 0	2 0 0	1 0 0	19 0 0	1 1 0	0 0 0	3 0 0	14 0 0	46
Phthalate NaOH pH 4.9	Potassium indole-butyrate	10^{-6} 10^{-7} Ck.	6 0 0	3 2 0	1 1 0	27 0 0	9 0 0	9 0 0	14 0 0	10 1 0	83
Nutrient buffer pH 5.2	Indole-butyric acid	10^{-6} 10^{-7} Ck.	4 0 0	5 0 0	11 0 0	5 0 0	9 0 0	10 0 0	14 0 0	10 0 0	68
Nutrient buffer pH 5.2	Potassium indole-butyrate	10^{-6} 10^{-7} Ck.	3 0 0	1 0 0	2 2 0	0 0 0	5 2 0	9 0 0	14 2 0	4 0 0	44
Column totals			18	13	18	51	27	28	47	39	
B. Neutral buffers pH 6.2–7.2											
Phthalate NaOH pH 6.2	Indole-butyric acid	10^{-6} 10^{-7} Ck.	0 0 0	1 0 0	6 0 0	36 2 0	3 4 0	20 1 0	28 1 0	44 0 0	146
Phthalate NaOH pH 6.2	Potassium indole-butyrate	10^{-6} 10^{-7} Ck.	3 0 0	4 0 0	3 0 0	4 5 0	4 0 0	22 0 0	22 2 0	9 9 0	87
KH_2PO_4 NaOH pH 7.2	Indole-butyric acid	10^{-6} 10^{-7} Ck.	17 0 0	3 0 0	3 0 0	34 0 0	6 0 0	19 0 0	3 2 0	5 3 0	95
KH_2PO_4 NaOH pH 7.2	Potassium indole-butyrate	10^{-6} 10^{-7} Ck.	8 0 0	11 0 0	1 0 0	1 0 0	17 0 0	1 0 0	21 0 0	30 0 0	90
Column totals			28	19	13	82	34	63	79	100	

* Summation of values for both varieties of tomato.
** Distilled water control.

shoots of the Briarcliff rose were used as a source of cutting material, indolebutyric acid in a concentration of 1 to 2.5 mg. per liter gave best results particularly from the standpoint of the subsequent shoot and root growth. Oliver recommended 20 to 40 mg. per liter for treating rose cut-

TABLE XIV

ROOTING RESPONSE OF TOMATO CUTTINGS ACCORDING TO THE NUMBER OF ROOTS FOR ALL
CONCENTRATIONS OF GROWTH SUBSTANCE AND ALL DILUTIONS OF EACH BUFFER
(DATA FROM TABLE XIII)

pH value of 10^{-3} M buffer	Indolebutyric acid			Potassium indolebutyrate			Class totals*
	Bonny Best	Marglobe	Group totals	Bonny Best	Marglobe	Group totals	
4.9	25	18	43	39	42	81	124
5.2	25	43	68	6	33	39	107
Column totals	50	61	111	45	75	120	231
6.24	44	100	144	18	66	84	228
7.23	56	38	94	21	68	89	183
Column totals	100	138	238	39	134	173	411

* Summation of values for acid and salt.

TABLE XV

ROOT FORMATION IN TOMATO LEAF CUTTINGS TREATED WITH ACIDIFIED UNBUFFERED
SOLUTIONS OF INDOLEBUTYRIC ACID. AVERAGE NUMBER OF ROOTS PER
CUTTING AT END OF SEVENTH DAY

Concn., mg./l.	pH adjusted with citric acid					pH adjusted with sulphuric acid				
	pH 3.9* 4.1**	pH 5.0 4.7	pH 6.1 6.8	pH 7.3 7.6	Group totals	pH 3.3 3.9	pH 4.8 5.4	pH 6.2 6.8	pH 7.4 7.6	Group totals
3.200	44	71	86	98	299	99	102	69	97	367
0.320	16	9	7	2	34	5	8	17	3	33
0.032	1	8	0	0	9	2	3	0	4	9
0.000†	1	2	0	0	3	0	0	0	0	0
Column totals	62	90	93	100		106	113	86	104	

* At beginning of treatment.
** At end of 24-hour treatment when cuttings were transferred to tap water.
† Tap water.

tings (73). Unlike Went's results with the pea hypocotyl, our results show that concentrations of growth substance ranging from 0.1 to 500 mg. per liter (0.5×10^{-6} to 2.5×10^{-3}M) will induce roots to form not only at the base of the cutting, but from the lower one-fourth to three-fourths of the stem. For this type of response there is no critical limit of 10^{-4}M such as Went has stipulated for the pea hypocotyl. Furthermore, many varieties of normal cuttings form roots in the middle portion of the stem, so that

Went's speculative explanations attempting to account for the absence of roots in the middle portion of the pea hypocotyl (114) are unnecessary assumptions for normal tissue.

Relative activity of acids, salts, and esters. Root formation tests with the tomato leaf cutting (Tables XIII, XIV, XV, and Fig. 5) and with *Hibiscus, Deutzia, Celastrus,* and grape (Tables II and XVI) show that

TABLE XVI

RELATIVE EFFECTIVENESS OF GROWTH SUBSTANCE ACIDS, SALTS, AND ESTERS AS DETERMINED BY THE AVERAGE NUMBER OF ROOTS PER CUTTING*

| Species and time taken | Concn., mg./l. | Naphthaleneacetic | | | | | Indolebutyric | | | | | Class totals** |
		Acid	K-salt	Methyl ester	Ethyl ester	Group totals	Acid	K-salt	Methyl ester	Ethyl ester	Group totals	
Deutzia	60	28	7	2	11	48	14	13	18	10	55	103
magnifica	40	15	6	6	6	37	16	6	8	8	38	75
(Jan. 19)	20	8	2	11	9	30	15	13	6	11	45	75
	0	2	2	1	2	7	2	2	1	1	6	13
Column totals†		53	21	26	32		47	41	36	34		
Hibiscus	60	0	2	6	4	12	10	17	10	12	49	61
syriacus	40	2	3	3	2	10	9	13	12	8	42	52
(Jan. 20)	20	0	0	0	1	1	5	10	12	6	33	34
	0	1	0	2	5	8	3	1	0	1	5	13
Column totals		3	6	12	14		27	49	41	31		
Celastrus	80	55	19	27	33	134	27	10	32	22	91	225
articulatus	60	24	21	19	17	81	20	14	11	38	83	164
(April 4)	40	15	10	23	21	69	27	10	10	13	60	129
	0	0	0	0	0	0	0	0	0	0	0	0
Column totals		94	60	74	82		74	41	57	88		
Concord	80	30	24	32	39	125	39	13	1	21	74	199
grape	40	16	19	31	0	66	22	32	26	35	115	181
(April 4)	0	4††	4	4	4	16	4	4	4	4	16	32
Column totals		50	57	73	50		65	59	34	69		

* Hardwood leafless cuttings when treated (4 cuttings per test).
** Summation of values for all forms of both growth substances.
† Corrected to give equivalent molecular values.
†† Average for 8 control cuttings.

salts of indoleacetic, indolebutyric, and naphthaleneacetic acids were of high activity, although not necessarily the same activity as the corresponding acids. On a molar basis (column totals, Table XVI) the difference is relatively small. The differences in the relative effectiveness of the two growth substances are also evident (column totals, Table XVI). This point has been given little or no consideration by other investigators who have confined their tests mainly to the use of indoleacetic acid. The high activity
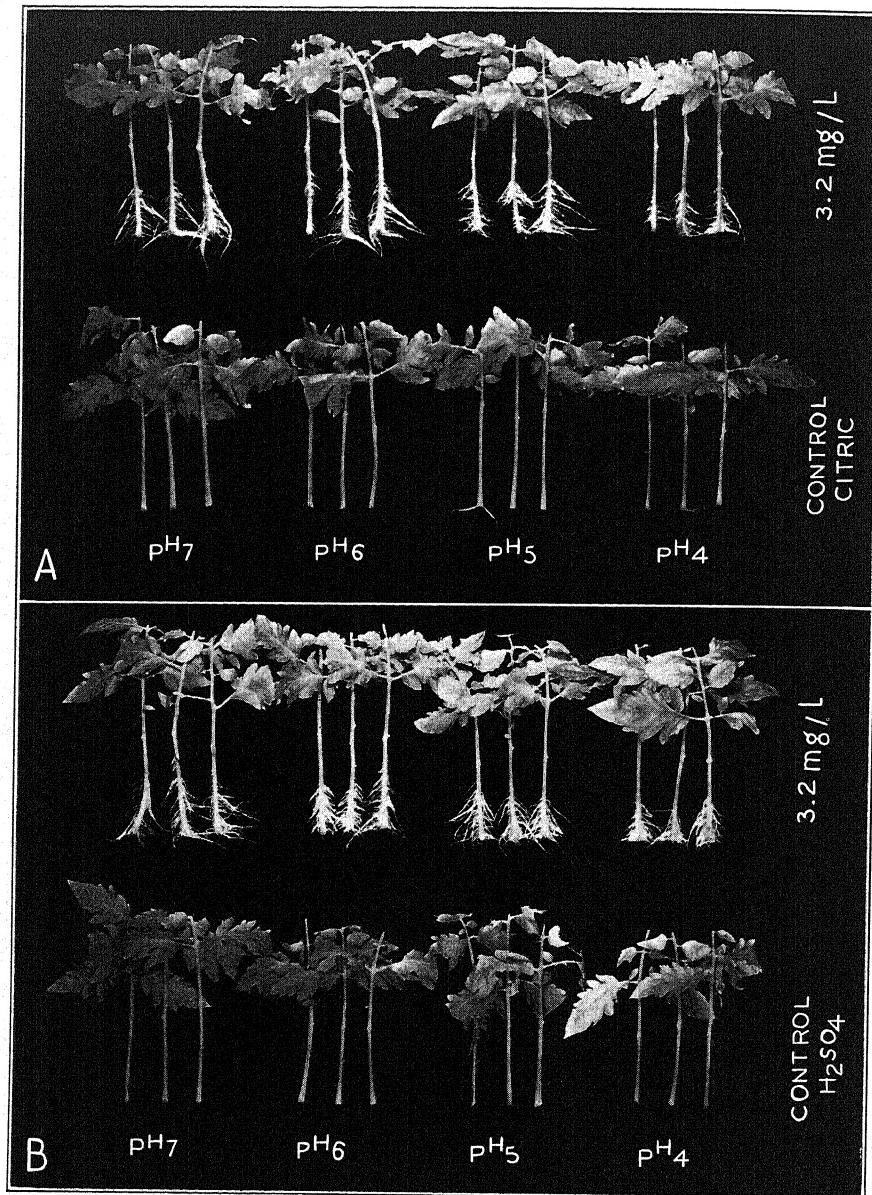
FIGURE 5. Root formation after eight days in tomato leaf cuttings treated 24 hours with indolebutyric acid adjusted to different pH values with (A) citric acid and (B) sulphuric acid (see text).

of indolebutyric acid and its potassium salt on *Hibiscus* is typical for many other genera. On the other hand, indoleacetic acid has always proved less effective for root formation than either naphthaleneacetic or indolebutyric acids which is in agreement with the results of others (13, 79, 88, 102, 103, 105, 106). It appeared to make little difference whether the acid was partially or completely neutralized with potassium hydroxide (Fig. 1). The possible objection that some preparations of growth substance salts have a distinctly acid reaction when dissolved in distilled water is therefore not a serious one for root formation except in those cases where a definite and

TABLE XVII

RELATIVE ACTIVITY OF ACIDS AND ESTERS FOR ROOT FORMATION IN TOMATO LEAF CUTTINGS AT END OF SIXTH DAY. AVERAGE NUMBER OF ROOTS PER CUTTING*

Form of growth substance	Name**	Bonny Best variety				Marglobe variety				Class totals†
		Water	10^{-6} M	10^{-5} M	Group totals	Water	10^{-6} M	10^{-5} M	Group totals	
Acids	IA	0	0	6	6	0	0	0	0	6
	IB	0	20	122	142	0	0	54	54	196
	NA	0	6	40	46	0	16	37	53	99
Column totals		0	26	168	194	0	16	91	107	301
Methyl esters	MIA	0	2	9	11	0	1	1	1	14
	MIB	0	0	116	116	0	1	82	83	199
	MNA	0	15	12	27	0	8	16	24	51
Column totals		0	17	137	154	0	10	99	108	262
Ethyl esters	EIA	0	2	12	14	0	1	24	25	39
	EIB	0	7	78	85	0	0	83	83	168
	ENA	0	12	32	44	0	0	42	42	86
Column totals		0	21	122	149	0	1	149	150	293

* Data for length of tissue from which roots emerged appear in Table XI.
** IA = indoleacetic, IB = indolebutyric, NA = naphthaleneacetic.
† Summation of values for both varieties of tomato.

known pH is wanted for experimental reasons. In the latter case it is of course preferable to prepare the salt from the particular acid used in the same tests, and to add that amount of alkali which will bring the solution to the desired pH.

The methyl and ethyl esters of indolebutyric acid were also of high activity for root formation in cuttings (Tables XI, XVI, and XVII). These results are an indication not only of the relatively high activity of the esters, but also of the differences due to the kind of growth substance and the specie of cutting.

The assumption that a growth substance can be active, or useful to the plant, only when applied in an acid solution on the basis that only under

such conditions can any appreciable quantity of the non-dissociated active form be available, had its origin particularly in the reports of Dolk and Thimann (24) and J. Bonner (8). Contrary to these stipulations, our results have shown that growth substances (other than auxins A and B) are highly active for growth promotion and root formation regardless of whether applied as an acid, salt, or ester (124). Avery and his associates (3) demonstrated that this held true also for the *Avena* curvature test. D. M. Bonner (7) and Thimann and Schneider (100) have recently confirmed our results relating to the relative activity of the acid and salt. Although under some conditions it appears that the salts may be more ac-

TABLE XVIII

pH VALUES OF GROWTH SUBSTANCE ACIDS AND SALTS IN DISTILLED WATER OF pH 5.97. MEASUREMENTS WITH GLASS ELECTRODE

Form of growth substance	Name of growth substance	Molar concentration			
		10^{-3*}	10^{-5}	10^{-6}	10^{-7}
Acids	Indoleacetic	3.79	5.67	5.92	5.94
	Indolebutyric	3.96	5.69	5.97	6.01
	Naphthaleneacetic	3.66	5.55	5.81	5.91
Neutralized acids	Indoleacetic	8.56	6.01	5.94	5.86
	Indolebutyric	9.10	6.01	5.87	5.87
	Naphthaleneacetic	6.16	6.04	5.86	5.96
Potassium salts	Potassium indoleacetate	5.59	6.01	5.91	5.94
	Potassium indolebutyrate	5.82	5.97	5.88	5.92
	Potassium naphthaleneacetate	6.09	5.94	5.86	5.86
Simple acids	Acetic acid	3.93	5.46	5.81	5.89
	Potassium acetate	6.87	5.96	6.01	5.96

* Stock solutions, not used in rooting tests.

tive than the acids, it was pointed out (124, p. 346) that this is of less importance at present than the fact that the salts are of high activity for growth promotion and root formation regardless of the conditions in the external solution which affect dissociation of the growth substance. For root formation in the tomato leaf cutting the activity of several growth substances had no direct relation to the pH of the solution or to the dissociation of the growth substance in either buffered or unbuffered preparations. Composition and concentration of the buffer, and not pH, determined relative effectiveness, and this held true only in the presence of the growth substance (Tables XIII and XIV). Although a minor influence of pH was observed, in contrast to the predominating influence suggested by J. Bonner (8), it was of particular interest that neutral buffers containing growth substance gave better rooting than the acid buffers containing growth substance (Tables XIII and XIV). This means that growth substances do not need to be made acid in order to obtain high activity for

root formation. Results in Table XII show that an acid reaction is not essential for growth promotion in the tomato leaf.

Considering the fact that Dolk and Thimann (24) had previously pointed out (p. 40) "The pH effect on the test is found principally in the buffered solutions; unbuffered solutions give much better results even in the alkaline range," it is surprising that J. Bonner failed to furnish comparable data for unbuffered solutions. Our measurements of the pH of unbuffered (Table XVIII) and dilute buffered preparations of growth sub-
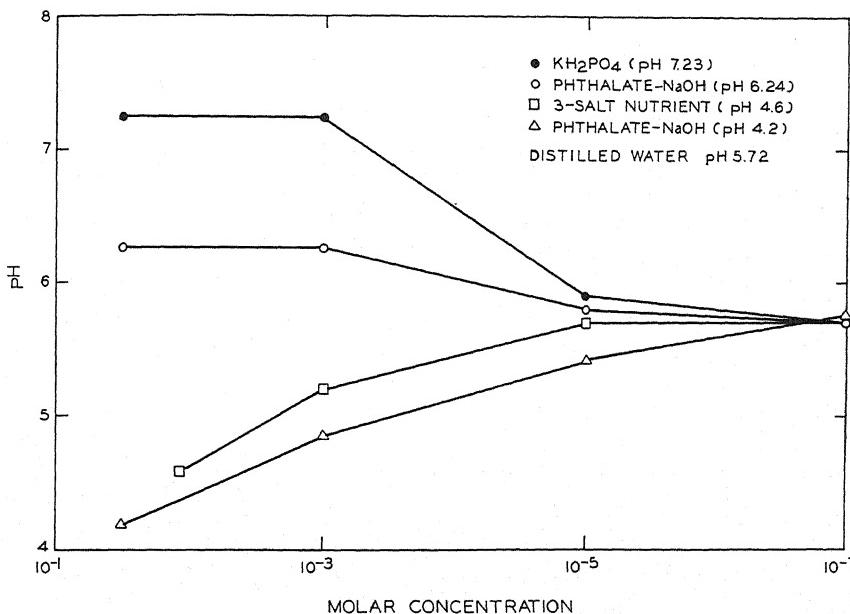
FIGURE 6. pH values of buffered solutions at different dilutions.

stance (Fig. 6) showed that at low dilutions (10^{-5} molar or less) there is no pH effect and hence the substance does not act as a weak acid. High activity is thus obtained under conditions of practically complete dissociation (1). Too much reliance cannot be placed on pH measurements of crushed tissue. There is the possibility that the pH of crushed *Avena* tissue does not represent the pH of cellular contents in intact tissue. As previously mentioned, Albaum and his associates (2) found that the pH of the cell contents of *Nitella* did not change when placed in buffers ranging from pH 4.1 to 7.1, even though the rate of penetration of indoleacetic acid was definitely a function of pH, being most rapid at the lower acid pH values. J. Bonner and others have given no consideration to the question of whether tissue other than *Avena* would have the same internal pH, and whether what was claimed for *Avena* would hold also for other plants. The

pH of fresh leaf extracts of *Cucumis sativus* L. was found to be 6.91 (glass electrode) and 7.22 (quinhydrone electrode). After 15 minutes the pH was 6.61 (glass electrode). Extracts of stems were more acid, giving a pH value of 5.6, and remained the same when measured at 3-, 6-, and 15-minute intervals thereafter (glass electrode).

That the pea hypocotyl test is not specific for known growth substances is indicated by the following statement (76, p. 36): "Buffers requiring citric acid were unsuitable since the citric acid contained traces of active substances which caused slight bending of the split stems." Since both Dolk and Thimann (24) and J. Bonner (8) used McIlvaine's phosphate-citric acid buffer, the marked buffer activity (over 50 per cent of the activity) which they reported may have been due to the active growth-promoting substances contained in the citric acid as pointed out by van Overbeek and Went (76). In our tests citric and sulphuric acids were not active for root formation (Table XV and Fig. 5). Likewise, citric acid in concentrations of 0.1 to 100 mg. per liter was not active for growth promotion in severed tomato shoots (geotropic bending), whereas the same concentrations of growth substance proved to be of high activity, as previously reported (125). Borriss (9) found salts and Brecht (12) found simple acids to be active for cell elongation. They see no reason for assuming that acid acts only through its effect on growth substance. The present results with buffered solutions (Tables XIII and XIV) show that for root formation the concentration and composition of buffers are important limiting factors independently of pH effects. Thimann and Schneider (100) have also shown how a salt such as KCl may become an important limiting factor so that their results confirm our conclusions with respect to the influence of buffer constituents in the presence of growth substance.

Neither D. M. Bonner (7) nor Thimann and Schneider (100) furnished data on which conclusions for a pH effect could be based. In D. M. Bonner's tests (7) the pH values of buffered and unbuffered preparations of growth substance were the same as distilled water (pH 6.05 to 6.2) and also the same as the pH reported for *Avena* tissue (8). Thimann and Schneider (100, p. 274) discussed the "Influence of Hydrogen-ion Concentration" without referring specifically to a pH value or pH measurement. In discussing the literature on pH effects, Thimann and Schneider (100, p. 274) make the following statement: "By contrast, the induction of root formation by auxin shows no effect of pH even with buffering (Went, 1934)." Reference to Went's paper (110) will show that no data are given on which a pH effect could be based and there is no mention of a single pH value or pH measurement. Furthermore, buffered preparations were not used as claimed in the above quoted statement. Having used unbuffered tap water preparations which had not been adjusted to any given pH value, Went assumed (110, p. 448) that it would be unnecessary to adjust

the pH or to add salts. Finally, the suggestion of Haagen Smit and Went (34, p. 853) that the present authors' results with lanolin preparations of growth substance represented merely an acid effect due entirely to pH and not to the growth substance, was not founded on any factual basis and they failed to note that similar results were obtained with a 1/25 stock solution containing 0.4 mg. per liter (37, p. 92). At this low dilution the acid can exert no pH effect; hence there was no possibility in these tests or in any other tests with low concentrations (1 p.p.m. or less) of changing the pH of the tissue as Haagen Smit and Went believed to be the case and as others have also claimed (8; 76; 99; 111, p. 173; 116).

The question of the influence of pH on growth is complicated by the many different angles from which the problem has been approached. Since the same growth substances which are active for cell elongation are also active for root formation, simple cell division, etc., it would be expected that pH might influence all processes in a similar manner. With respect to root formation the results obtained in our laboratory indicate there is no limiting influence of pH such as that claimed for cell elongation. In contrast to etiolated test objects such as the *Avena*, which exhibit only growth promotion, our test objects exhibit growth promotion, root formation, proliferation, and the inhibition of bud growth—that is, most of the responses known to be induced by growth substances. Since similar responses are induced in the same test object by growth substance acids and their corresponding salts and methyl and ethyl esters independently of the pH of the preparation applied, it is difficult to explain the results solely on the basis of the dissociation hypothesis. Our results with green tissue are likewise not explainable on the basis proposed by Went and Thimann (116, p. 99) of the excessively high ("unphysiological") concentrations used, since in the case of the tomato, effective concentrations of growth substance range from 1:100,000,000 to 1:10, depending upon the method of application.

In our tests with green tissue, growth promotion and root formation were not induced by simple acids nor were they induced by acid buffers minus growth substance, although a noticeable influence of buffer components was observed in the presence of growth substance (Tables XIII and XIV). There is the further unlikelihood that in green tissue, such as the tomato, the pH of the cell contents is lowered in tissue several inches beyond the region treated, or which is beyond the surface in contact with the growth substance preparation. Van Santen (82, p. 515) emphasized the importance of intimate contact between the external solution and the cell contents of *Avena*, if the pH of the tissue were to be lowered, which she accomplished by continuous aeration of the solution. However, in her tests the pH of the contents of *Avena* cells was not measured. Opposed to van Santen's suggestion, that acid buffers would lower the pH of plant tissue

in general, are the results which we have obtained independent of pH or acid effects and those reported by Albaum *et al.* (2) in which case the pH of the cell contents of *Nitella* remained unchanged when left in contact with acid buffers ranging from pH 4.1 to 7.1

Transport of applied growth substance. In tomato leaf cuttings and in cuttings of other plants the absorption and upward movement of the basally applied growth substance were indicated by the increase in number of roots formed and in length of tissue from which roots emerged according to the increase in concentration of the growth substance. Results for *Chrysanthemum* (Table X) are representative of those obtained with other genera. The influence of transpiration on root formation (Tables VII, VIII, and IX) becomes evident only when the atmospheric conditions are greatly different. These results are in agreement with those previously reported (42).

The movement of indolebutyric acid upward in one-half of a split tomato petiole and downward in the other half was according to the location of the source of the supply as indicated in Figure 4 B. Similar results were obtained with split stem cuttings of *Hibiscus* in which case a concentration of 40 mg. per liter was used. Special cases of upward transport to the terminal bud, illustrated for *Euonymus* in Figure 7, were obtained with many other species of plants such as *Malus* (flowering crab), *Corylus*, *Ilex*, *Cornus*, *Viburnum*, *Rosa*, *Celastrus*, *Cotoneaster*, *Azalea*, and *Chrysanthemum*. The concentration which will induce this type of root formation varies with the kind of plant, the duration of treatment, and the age and relative activity of tissue used for cuttings. Concentrations of indolebutyric acid ranging from 0.1 to 10 mg. per liter induced root formation in tissue from the base of the cutting upwards for a distance of one to several inches in types which normally root only at or near the base, such as certain varieties or species of rose, *Chrysanthemum*, Kurume *Azalea*, *Cornus*, *Ilex glabra*, *Hydrangea*, *Forsythia*, *Lonicera*, *Ageratum*, *Buddleia*, *Callicarpa*, *Dianthus* (carnation), Concord grape (softwood), *Coleus*, *Deutzia*, *Euonymus*, *Franklinia* (*Gordonia*), *Halesia*, *Hedera*, *Morus*, *Petunia*, *Philadelphus*, *Prunus* (ornamental), *Spiraea*, and *Weigela*. The following cuttings required a concentration of indolebutyric acid greater than 10 mg. per liter to induce rooting for any appreciable distance upward from the base: *Azalea mollis*, *Acer palmatum*, *Buxus*, *Catalpa*, *Chamaecyparis*, *Clematis*, *Cryptomeria*, *Dahlia*, *Daphne cneorum*, *Gardenia*, *Corylus*, *Hibiscus*, *Ilex opaca*, *I. cornuta*, *I. aquifolium*, *Juniperus*, *Kolkwitzia*, *Syringa vulgaris* varieties, *Magnolia soulangeana*, *Pachysandra*, *Ligustrum*, *Sambucus*, and Concord grape (hardwood).

All species of the same genus did not respond alike to a given treatment, and the same may be said of different varieties of the same species. *Ilex glabra* was much more sensitive than *I. opaca* and *I. cornuta*. The Briarcliff
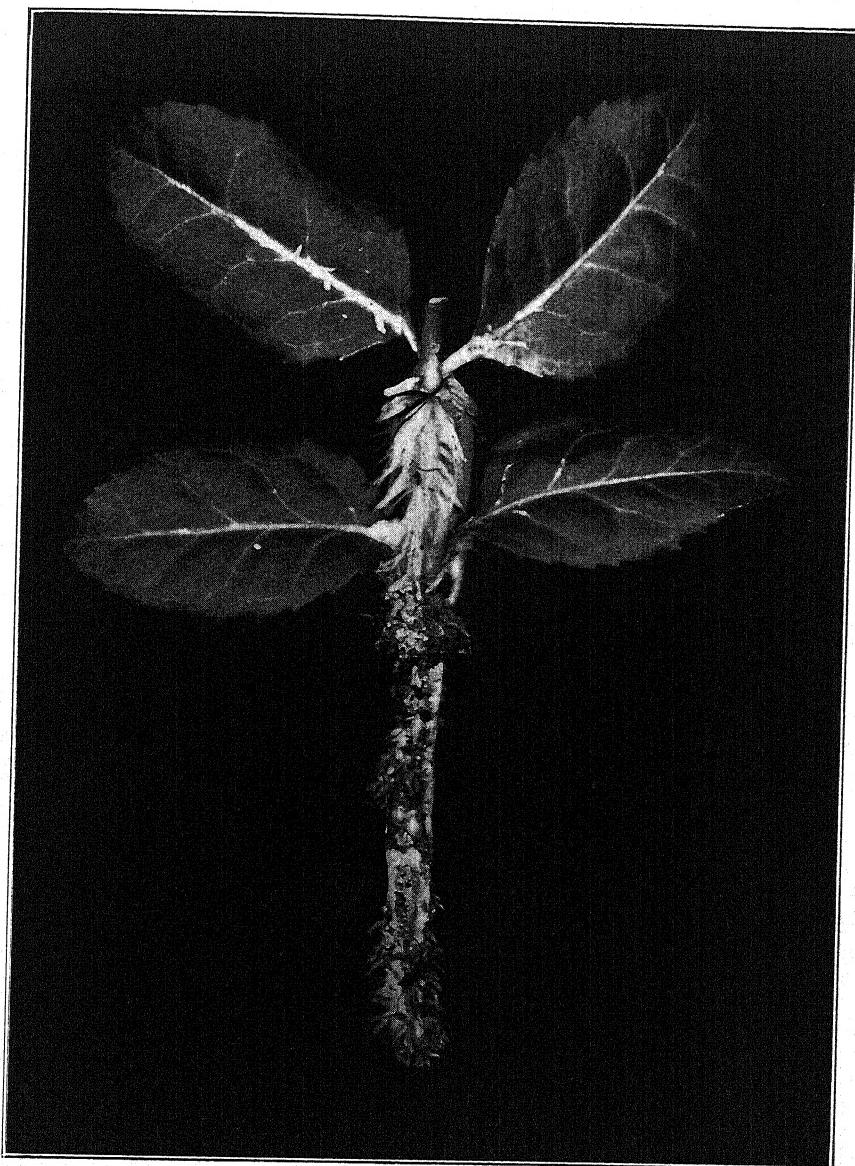
FIGURE 7. Cutting of *Euonymus radicans* 17 days after lower one-third was immersed for 24 hours in a solution of indolebutyric acid (160 mg. per liter). Note roots on stem above uppermost axillary buds and on upper leaves. Cutting covered with cheesecloth while in rooting medium.

rose was more sensitive than Paul's Scarlet and Souvenir Claudius Pernet. A concentration of 1 to 2.5 mg. per liter was optimum for root formation in the Briarcliff rose cuttings. Higher concentrations generally retarded bud growth for periods of one to several weeks and caused injury to the stems of some of the cuttings. In comparison, Oliver's recommendation of solutions containing 20 to 40 mg. per liter is excessively high for roses (73, p. 387). Greenwood cuttings of the Concord grape were more sensitive than hardwood leafless cuttings. Both types of cuttings rooted profusely from the base upwards. A 24-hour treatment with concentrations within the range of 0.1 to 100 mg. per liter has proved effective for root formation in all of the more than 100 species which have responded to basal application of indolebutyric acid. The assumption by Went (113, 114) that polar (downward) transport disappears when concentrations of growth substance of 10^{-4} M or higher are applied to cuttings does not hold true for the cuttings used in our tests. In the tissue used as test objects in our laboratory, there has been no evidence of a polar (downward) transport of applied growth substance; hence, a polarity which did not exist could not disappear.

Considering some of the data used in support of the polarity concept, it is apparent that if there is less or only a slight movement upward, as compared to downward movement, the data are interpreted as showing a strictly basipetal polar transport. Examples of this are furnished by Beyer (6, p. 330, 339), van der Weij (107, p. 445–449), Went (109, p. 536, 537), and Nagao (70, p. 726). In contrast, there are many reports based upon similar tests which show that the transport of growth substance is not basipetally polar, notwithstanding that in many cases there may be a more pronounced or a more rapid movement in one direction (27, p. 459; 28; 51, p. 115; 52; 53; 57; 65; 80; 90; 91; 92; 93; and the reports of Laibach and his associates 59, 61, 62, 63). Otte (75, p. 106) remarks that the "leakage explanation" (assumed movement of growth substance along external surface of test object) to account for observed upward transport, used by Beyer (6) and van der Weij (107), is not supported by the results of other workers. The results which Went obtained with dyes (109), and Jost and Reiss with solutions of growth substance (52), were regarded by Clark as proof that there is a "semi-polarity" of auxin transport (14, p. 410). Clark (14, 15) had already demonstrated that there was no relation between electrical polarity and auxin transport as claimed by Went (109). Our results (42) showed that growth substance could be transported simultaneously upward and downward in or external to the transpiration stream —a fact which appears untenable to Cooper (17) and Ferman (28, p. 282). Gustafson and Darken found that phosphorus was conducted upward in both the xylem and the phloem (33). Clements and Engard (16) have pointed out that girdling experiments are generally unreliable for demon-

strating whether or not there is any transport of solutes in the xylem, due to the damage which results from girdling. Cooper (17) has not considered this effect. As further evidence that organic crystalline substances are transported both upward and downward and in some cases to nearly all parts of the plant, the crystalline tobacco virus may be cited (48). At least it may be said with certainty that plants are provided with the mechanism for conducting substances simultaneously to different parts. It is thus unnecessary to postulate a complicated mechanism whereby certain substances (calines) move only upward and others only downward as Went has done (114, 115). Ferman (28, p. 282) has considered Went's idea "superfluous." Propagators are familiar with the fact that many cuttings taken after January 1 will form shoots several inches in length before roots appear. We have found that when these new shoots are cut off, they root in a normal manner. There is thus no reason for assuming that substances essential for shoot growth must be sent directly from the roots. Many substances important for growth are stored in the aerial parts of plants.

Some of the limitations of the *Avena* curvature test were discussed by the authors previously (124), and also by Avery *et al.* (3), Scheer (83), and du Buy (26). Considering the unreliability of the *Avena* curvature test for measuring the concentration and transport of known substances, it is obvious that the method is still less suitable for measuring either the concentration or transport of natural or applied growth substances in other plants. The erroneous assumption that the distribution and transport of growth substance in other plants can be measured in terms of *Avena* curvature values has led to many misinterpretations and much confusion. Results reported recently by Skoog (89) are an illustration of this fact. In the first place, no consideration was given by Skoog to the known difficulties encountered while attempting to extract growth substance quantitatively from green tissue, either by chemical reagents or by diffusion into agar (26, 94). The excess growth substance recovered may represent only a small part of that actually present and that which is used in growth processes obviously cannot be recovered.

Skoog observed that analysis of certain tomato segments gave negative curvatures on *Avena* (89, p. 371), but still maintained that "Failure to obtain auxin . . . was not from a lack of polarity in these tissues." He has assumed that the unidirectional transport of tomato and squash diffusates in *Avena* stumps constitutes evidence of the transport of these substances in the treated plants. His evidence (89, p. 371) "Clearly the present results are in complete agreement with the earlier work on polar transport" can thus apply only to the assumed polar transport in *Avena* and has no direct bearing or known relationship to the transport of natural or applied growth substances in tomato and squash. In a like manner, his explanations and conclusions with respect to distribution of growth substances,

channels of transport, and the influence of various factors such as trans-
spiration, pH, etc., were erroneously claimed to be true for tomato and
squash. Actually, the explanations can apply only to *Avena* which was
used as the basis of all tests. It is to be noted that Skoog furnished data
only on the influence of transpiration on upward transport but com-
parable data were not given on downward transport and hence there
was no factual basis for assuming that the rapid downward transport
(20 cm. per hour) was independent of transpiration and consequently out-
side of the xylem. In contrast to the indirect method used by Skoog to
determine transport, results reported from this laboratory and also by Lai-
bach and his associates (30, 59, 60, 61, 62, 63, 64) have been based on
measurable growth responses in the green tissue of treated plants. The
criticism which Skoog makes (89, p. 371) of cases where unjustified gen-
eralizations are made "from experiments of one kind to other kinds done
under completely different conditions" is exactly what he has done in gen-
eralizing from results of transport in *Avena* to the type of transport as-
sumed to occur (but not determined) in tomato and squash. In addition,
no mention was made of a growth response in the treated plants which
constituted the basis of our tests, and hence none of his tests was com-
parable to ours. Reference to agreement or disagreement with our results
must be judged from this standpoint. Data reported by Harrison (35) in-
dicate that anatomical changes may be used to demonstrate the probable
channels of transport which for *Iresine* appeared to be in the xylem rather
than in the phloem. Laibach and Fischnich (63, p. 87–88) have suggested
that transport occurs in different channels according to the kind of plant
—that is, in xylem, phloem, or parenchyma.

Skoog (89, p. 367) and Cooper (18, p. 604) have assumed, without hav-
ing determined experimentally, that when cuttings or plants are in a high
relative humidity during treatment, there is little or no absorption of the
basally applied growth substance. In our tests measurable absorption oc-
curred when cuttings were placed under a bell jar (Table XIX). Other tests
showed that the increase in root formation was not proportional to the
increased absorption at lower humidities (Tables VII and VIII). The rela-
tive intensity and the quality of light under glass filters also influenced
root formation (Table IX). The physiological method (root formation) for
determining that absorption has occurred in the treated plant, is believed
to be more reliable and a more sensitive test than those used by Skoog (89)
and Cooper (18) for measuring extractable growth substance on another
plant. Cooper's conclusions are invalid for root formation in lemon cut-
tings, since they were based on cell elongation tests in *Avena*. The existence
of a hypothetical root-forming substance (rhizocaline) should be verified
by root formation tests and not by cell elongation tests with *Avena*. In our
tests (Table XX) the root-forming activity of growth substance extracted

TABLE XIX

AMOUNT OF SOLUTION ABSORBED BY LEAFLESS CUTTINGS (5 BALDWIN APPLES AND 5 BARTLETT PEARS) DURING 24 HOURS UNDER DIFFERENT ATMOSPHERIC CONDITIONS. CUTTINGS TREATED WITH INDOLEBUTYRIC ACID

| Concn., mg./l. | Loss by weight (g.) | | | | Loss by volume (cc.) | | | |
| | With cuttings* | | Without cuttings** | | With cuttings* | | Without cuttings** | |
	Open†	Closed††	Open†	Closed††	Open†	Closed††	Open†	Closed††
40	8.2	2.8	—	—	11.0	6.0	—	—
Water	8.3	2.1	5.2	0.5	11.0	5.0	5.5	1.0

* Determined by weighing beaker, containing solution and cuttings, before and after 24 hours.
** Represents loss in weight due to evaporation from free surface.
† Exposed to laboratory air.
†† Placed under 2-liter enamel beaker lined with moist paper towel. Double layer of moist cheesecloth placed over tops of cuttings.

from treated cuttings was tested for root formation in similar untreated cuttings. In more recent tests described in the next section the tissue extract was active for growth promotion and root formation in the same test object (tomato stem cuttings).

TABLE XX

ROOT FORMING ACTIVITY OF GROWTH SUBSTANCE EXTRACTED WITH CHLOROFORM FROM BASES OF 20 TREATED TOMATO LEAVES AND APPLIED TO NON-TREATED TOMATO LEAF CUTTINGS

| Dilution of extracted growth substance* | Av. No. roots per cutting | |
	Treated bases	Control bases
Diluted to 1/2	28	7
Diluted to 1/4	8	3
Control (water)	4	6

* Residue (chloroform extraction method) made up to 50 cc. and then diluted as indicated.

The postulation of several hypothetical substances (calines) for regulating growth (114, 115) is an attempt to account for upward movement of growth substances without appearing to reject the polarity concept. In a like manner, the terms food factor, primary factor, primary activity, etc. (116) are merely another way of saying that growth is not a simple process controlled directly by a single substance—"auxin, the plant growth-hormone" (111). The idea that a single substance directly controls all important physiological responses (cell enlargement, cell division, differentiation, correlation, etc.) by "attraction" (17, 18, 28, 111, 115, 116) or other unknown mechanisms, is another way of stating that physiological processes make use of many important substances—not in a single step, but in sev-

eral steps—which was a conception well known before the advent of growth substances. Went's axiom "without growth substance, no growth" has been difficult to prove, due to the limitations of present experimental methods (82) and is essentially no different from that proposed by Avery *et al.* (4, p. 557) "no nitrogen, no growth," and could be said of other important substances such as P, K, C, Fe, etc. There is the further difficulty that certain growth responses (e. g., geotropism) appear to induce the formation of growth substance as indicated by the results reported by Schmitz (86), Oortwijn Botjes (74), and the authors (125). Söding also suggested that the relation of growth substance to growth might be represented in the following way: growth substance—growth—growth substance (95, p. 302).

At least it may be said that the marked specificity of growth substance action claimed by the earlier workers has been drastically modified to the point where specificity is the exception rather than the rule. Whereas it was earlier specified that a growth substance moved only downward, but "never in the opposite direction" (108, p. 205), attempts are now being made to explain how and why a growth substance moves upward, according to some mysterious "attractive" force (114, 115). That growth substances move upward has been known at least since 1925 (90).

The geotropic response was used in recent tests (125) as a means of determining the relative effectiveness of different substances for inducing growth as evidenced by the increase in surface markings, regulating the direction (negative or positive) and rate of geotropic bending, and as evidence of transport (125). Epinasty of leaves occurred on the upright portions of stems treated with the minimum effective concentration (0.1 mg. per liter) which induced a measurable increase in growth (surface markings on stems) and a faster rate of bending. A similar epinasty of leaves was induced, without the application of growth substance, on plants subjected to a geotropic stimulus and then placed in an upright, vertical position. These results indicate that known growth substances applied to the tomato in concentrations of 0.1 to 1 mg. per liter induced growth responses similar to those induced by natural growth substance in the untreated tomato. Thus the unequal distribution in the shoot of naphthaleneacetic, indolebutyric, and indoleacetic acids appeared to be the same as that for the natural growth substance of tomato. Each of these three substances could also function the same as, and be substituted for, natural growth substance in causing geotropic bending in plants which had failed to respond to gravity due to loss of their natural growth substance when the plants were kept in the dark (123, 125). In this case, geotropic bending was induced when the active growth substance was applied at the tip or at the base of intact plants or severed shoots—thus indicating a non-polar type of transport for the applied growth substance. These results were con-

firmed by Oortwijn Botjes (74). The most convincing evidence that applied growth substance moves longitudinally in either direction was indicated in recent tests whereby the applied indole substance was extracted from treated plants and detected as an indole compound according to the Winkler and Petersen test (117).

Detection of applied growth substance in treated plants. A positive test for indole compounds according to the Winkler and Petersen method was obtained in extracts of tissue taken from different parts of treated plants 3 hours to 24 days after treatment with aqueous solutions or lanolin preparations of indolebutyric or indoleacetic acids. Control tissue did not give a positive reaction. Although tissue extracts from treated plants gave a positive indole test, this constitutes evidence only of the presence of an intact indole nucleus. However, the brilliant reddish-violet color obtained with treated tissue extracts was similar to that obtained with prepared aqueous solutions of indoleacetic and indolebutyric acids and was readily distinguishable from the deep reddish coloration obtained with prepared solutions of indole or the characteristic bluish-violet color of tryptophane solutions. This is in agreement with the differences described by Winkler and Petersen (117, p. 211). Regardless of whether the substance detected in extracts from treated plants was the indole compound applied, the results indicate that the applied growth substance was transported in a form which contained an intact indole nucleus. By this method the presence of applied growth substance was detected one to six inches above and below the region of tomato stem treated with lanolin preparations or aqueous solutions of indolebutyric or indoleacetic acids. Treated gladiolus corms gave a positive test 24 days after treatment, tomato leaf cuttings after 7 days, and cuttings of *Lonicera maacki* after 17 days. It was not determined how long after treatment a detectable quantity of applied indole substance remained in the tissue.

The ether extract, obtained according to du Buy's method (26), from treated plants which gave a positive indole test, was also active physiologically for growth promotion (epinasty) and root formation in tomato stem cuttings. By the same method, geotropically stimulated tomato shoots (treated with indolebutyric acid), yielded extracts from the lower side which contained considerably more indole substance (at least three times) than extracts from the upper side, as judged by the indole ring test, the indole colorimeter determination, and according to the relative number and length of roots induced in tomato stem cuttings. Since the applied indolebutyric acid was detected (as an indole compound) in shoots and roots which developed after treatment of gladiolus corms, it is evident that the applied growth substance was relatively stable (in a physiologically active form) in the tissue, and was transported acropetally from treated corm tissue into newly formed non-treated shoots and roots.

Exudates from cut portions of tomato stems and leaves also gave a positive test for the applied growth substance and were active for growth promotion and root formation in tomato cuttings. These results confirm our previous conclusion that transport of applied growth substance is not basipetally polar (42). The test for indole compounds was sufficiently sensitive to detect indolebutyric acid in a concentration of 1 to 2 mg. per liter and indoleacetic acid in a concentration of 3 to 5 mg. per liter. The latter value agrees with that reported by Winkler and Petersen (117, p. 211). The quantity of growth substance in the volumes of solution tested (0.01 to 2.5 cc.) ranged from 0.00001 to 0.0025 mg. With respect to transport. it thus appears that applied growth substances such as indolebutyric and indoleacetic acids move upward and downward in plant tissue.

Data relating to the detection of applied indole compounds indicate not only that the indole nucleus is relatively stable, but also that there is presumably not a sufficient quantity of a naturally occurring indole compound to give a positive test (in control tissue extracts) by the same method. However, it is a matter of interest that one of the fractions discarded during the extraction procedure (insoluble in water, alcohol, and ether) contained a substance which, after digestion with concentrated acid or alkali, gave a positive indole test. Both control and treated tissue contained this substance. It seems likely that this fraction contained a tryptophane-protein complex which yielded free tryptophane following digestion with acid or alkali. If this assumption be true, it means that the indole test may be used for detecting applied indole compounds in the free state independently of the naturally occurring indole compounds which, in the tomato, appear to be present in a complex form with proteins.

Previous methods for demonstrating that transport of applied growth substance is not unidirectional (42) account for the growth substance used in the growth responses induced, and the present method accounts for the excess growth substance which can be detected by the Winkler and Petersen test. In contrast, analysis of tomato tissue by the *Avena* method does not account for growth substance used in growth processes (in the tomato), and does not furnish a means for detecting the substance as that which was applied to the treated tomato plant. Results obtained by Skoog (89) and Cooper (18) and the conclusions derived from them must be considered from the standpoint of the unreliable *Avena* method which they used as a basis for determining the action of growth substance in the tissue of other plants. In their tests, negative values cannot be regarded as evidence that the applied growth substance did not move to or through the tissue analyzed.

Identification of applied indolebutyric acid. Basal ends of severed tomato shoots 10 cm. in length were treated with aqueous solutions of indolebutyric acid. The exudate collected from the distal cut ends during a period of
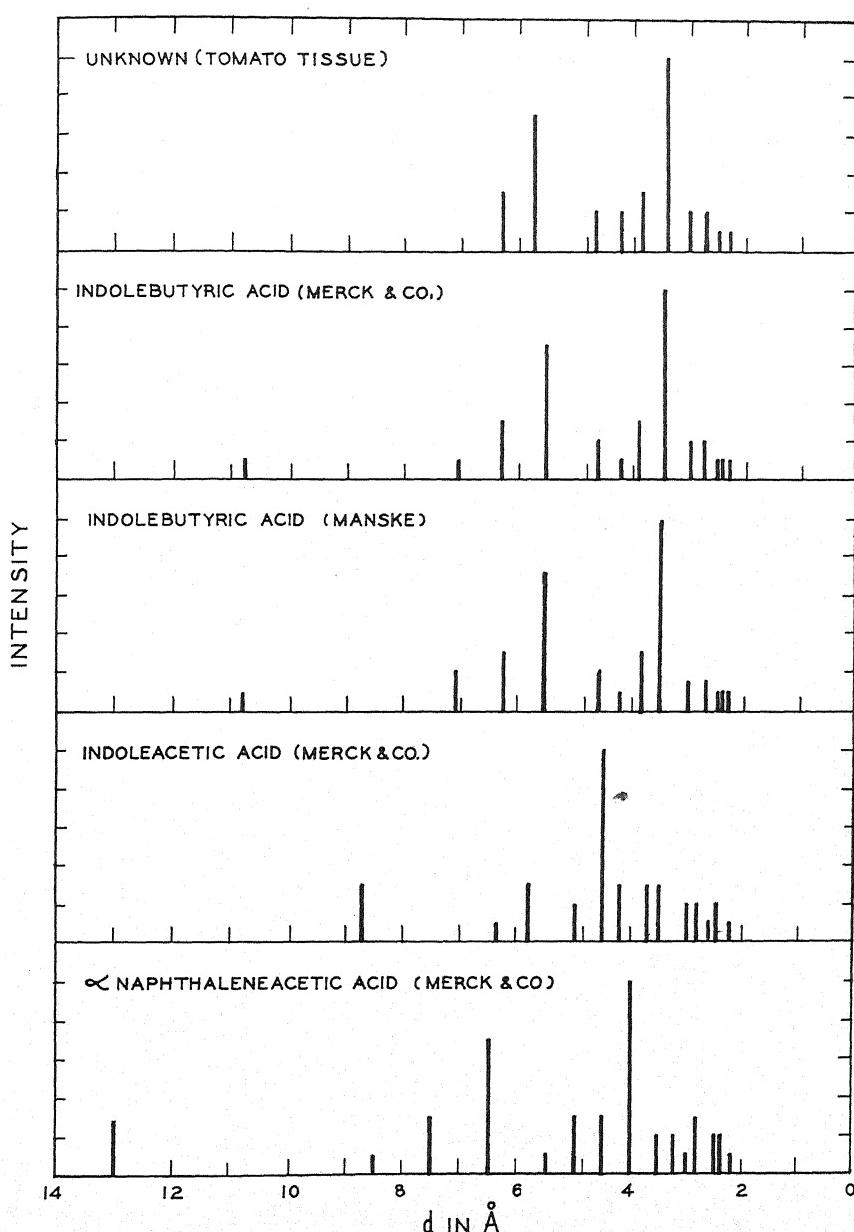
FIGURE 8. Measurements for X-ray diffraction patterns of unknown and known crystalline growth substances.

16 hours was analyzed for growth substance activity according to methods referred to in the preceding section (indole nucleus and physiological tests, both being positive) and also for X-ray diffraction characteristics. The procedure for extracting the growth substance was as follows: the exudate was acidified with HCl to pH 2.5 and shaken with ether for 3 hours. The ether layer was separated and added to an equal volume of 0.05 M sodium carbonate and shaken for 3 hours. The aqueous layer was separated, acidified with HCl to pH 2.5, added to 1/3 its volume of ether, and shaken for 3 hours. Separation and evaporation of the ether fraction (almost to dryness) yielded an aqueous residue which was dissolved in 95 per cent ethyl alcohol. The alcoholic solution was placed at −11° C. for 4 days. The mother liquor was then decanted, and the crystalline residue was dried and used, without grinding, for X-ray analysis. All other samples, for which data appear in Figure 8, were finely ground.

The X-ray diffraction pattern for the unknown sample obtained from treated tomato shoots was similar to that for indolebutyric acid. Measurements[1] for these patterns appear in Figure 8. Considering these results and the fact that the diffraction patterns for indoleacetic, indolebutyric, and naphthaleneacetic acids are distinctly different (Fig. 8), it would appear that X-ray analysis offers new possibilities for determining the presence of, and the form in which natural and applied growth substances occur in plant tissue.

CONCLUSIONS AND SUMMARY

Substances highly active for root formation in intact plants and in cuttings of herbaceous and woody types were tested for root-forming activity in leaf cuttings of the tomato. Important factors which influenced root formation were the kind of growth substance, the species or variety of cutting, the age and relative activity of the tissue used for cuttings, relative amount of leaf surface, and the atmospheric conditions (light, temperature, and humidity) during treatment. Indole and naphthalene compounds were more effective than phenyl compounds. Simple acids (citric, acetic, and sulphuric) were not active for root formation. Thus, claims repeatedly made in the literature, that a very few substances are specific for growth promotion but not for root formation, were not substantiated by our results.

Within a limited range of concentrations, growth substance preparations containing ethyl, methyl, and iso-propyl alcohols increased root formation. Above this range alcoholic preparations were toxic, but the alcohols themselves were not active. It is believed that solubility and permeability relationships were changed so as to facilitate the penetration of the growth substance.

[1] All X-ray diffraction data were furnished by W. A. Sisson of this Institute.

Retreatment of cuttings at the end of 24 hours, after removal of a basal portion one-half to two inches in length, induced the formation of more roots than in similar cuttings not retreated. These results are in agreement with those reported by Hellinga, Pearse, and Dorfmüller, but are in disagreement with those of Cooper who claims that the first treatment depletes the tissue of a hypothetical root-forming substance. It would thus appear that data relating to retreatment cannot be used in support of the "local mobilization" hypothesis as claimed by Cooper and by Went.

Results with the retreatment of intact cuttings (bases not removed) from several weeks to several months after an initial treatment with growth substance, indicated that there was no additive effect of the two treatments. In this case the controls (not treated with growth substance initially) responded to treatment at a later period, like cuttings which had received the initial treatment with growth substance. These results confirm those reported by Stuart and Marth relating to "deferred" treatment of *Ilex* cuttings, but differ from those of Cooper and Went who reported a favorable effect only for cuttings which had been treated previously.

Increasing the concentration of substances highly active for root formation caused a noticeable increase in the number of roots formed in cuttings. In a like manner the length of tissue from which roots emerged was also a function of concentration. Substances which showed only slight or questionable activity for root formation did not exhibit similar close relationships between concentration and rooting response. The tomato leaf cutting test appears to be the most sensitive and the simplest test reported for root formation.

Salts and esters of growth substance acids were found to be of high activity although not necessarily of the same activity as the acids for root formation. The pH of the solution was not an important limiting factor within the range of pH 4.2 to 7.2 either in the case of buffered or unbuffered preparations which, in the absence of growth substance, were not active for root formation. Since growth substances were active for growth promotion and root formation at concentrations of 1 mg. per liter or less in buffered and unbuffered solutions, and under conditions in which there would be little or no free acid, the dissociation hypothesis has no application to root formation or growth promotion in green tissue. The assumption that the pH of cell contents of plant tissue in general is readily lowered (without injury) by acid buffers and thereby accounts for the effect of acid on growth, appears unlikely in our tests in which there was no provision for attaining an equilibrium between cell contents and the external growth substance solution either locally or at distances up to several inches from the treated region.

Results with tomato leaf cuttings and stem cuttings of more than 100 species of commercially important plants confirm our previous conclusion

that applied growth substance is transported upward and downward. The basal immersion method for treating cuttings, which is now the method used in practice, was based upon the principle that growth substances are absorbed readily through the basal cut surface and are transported upward. This view is not only in direct opposition to the earlier "apical" method of treating cuttings (recommended by Went), but is also opposed to Went's "local mobilization" hypothesis which stipulates that basally applied growth substance acts only by "attracting" toward the basal end, all substances essential for root formation so that the applied growth substance itself is not transported upward. The further stipulation that upward transport can occur only under special conditions and only at concentrations higher than 10^{-4} M, has been definitely disproved both by our results and by those of other workers which show that upward transport occurs at concentrations ranging from 2×10^{-8} M to 2×10^{-4} M.

Substantial evidence that the applied growth substance moved upward in cuttings, and both upward and downward (in intact plants and severed shoots) from regions treated either with lanolin or aqueous preparations, was furnished by data in which the applied indole compound was detected in different parts of the treated plant by means of the Winkler and Petersen indole test. With the same method, a positive test was obtained in treated tissue up to 24 days after treatment. The extract of tissue from treated plants, but not in the case of control plants, which gave a positive indole test, was also active physiologically for growth promotion and root formation in tomato stem cuttings and for root formation in tomato leaf cuttings. Geotropically stimulated tomato shoots treated with indolebutyric acid yielded extracts from the lower side which contained about three times as much indole substance as extracts obtained from the upper side. This quantitative difference was verified by physiological tests on tomato stem cuttings. These results are not only in disagreement with those of Skoog who based his conclusions on transport in *Avena* and not in the tomato as he claimed, but they indicate that the use of *Avena* curvature values is not a reliable criterion either for the action or for the transport of growth substance in the tissue of other plants. The sensitivity of the indole test was 1 mg. per liter for indolebutyric acid and 3 mg. per liter for indoleacetic acid. The minimum quantities of these two growth substances which could be determined ranged from 0.00001 to 0.0025 mg., depending upon the actual volume of the solution tested. Results with the indole test indicate that the indole nucleus is not readily destroyed and remains for many days in a form which is physiologically active. By definite detection of applied growth substance in treated plants, it is believed possible to determine the validity of many explanations for the action of growth substance which have been based on indirect evidence or on assumptions. The final step in identifying the structure of the applied growth

substance (indolebutyric acid) by means of X-ray analysis indicates that this method offers new possibilities for determining the presence of, and the form in which natural or applied growth substances occur in plants, and the direction and rate of their translocation.

The tomato has proved useful for determining how natural and applied growth substances influence growth, differentiation, and correlative responses in green tissue. Unlike etiolated test objects such as *Avena, Pisum, Helianthus,* etc., the tomato is readily adapted for experimental study of all important physiological responses characteristic of growth substances. It is believed the tropic response of severed tomato shoots constitutes one of the most valuable and one of the most sensitive tests for determining substances which act as, and may be substituted for, the natural substances redistributed under the influence of gravity and light in green tissue. The modification of this test whereby the green tissue is depleted of natural substances which induce tropic responses, by a pre-dark treatment, makes it possible to determine which substances are highly active, and also to distinguish between those which are active and those which are not. From the standpoint of specificity and sensitivity, the tomato tropic test compares favorably with the *Avena* curvature test, but unlike the *Avena,* the tomato does not respond to simple acids (acid growth effect) and does not exhibit basipetal polar transport as claimed by Skoog. However, if we consider the results obtained with all test objects, there is much less specificity of growth substance action than has been claimed by the Utrecht school. Data continue to accumulate in support of the idea that the growth substances known to date act as chemical stimulants in the sense postulated by Fitting.

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STUDIES
WITH SEEDS AND GERMINATION

By
WILLIAM CROCKER

Studies with Seeds and Germination

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Professional Paper No. 29, Boyce Thompson Institute for Plant Research, Inc.
Yonkers, N. Y.

THERE are three questions that come into our seed laboratory repeatedly about one or another sort of seed. These questions are asked, of course, by practical producers, handlers or users of seeds. How shall we treat a certain kind of seed in order to get prompt, complete and simultaneous seedling production? How can we make quick viability tests of seeds that are much delayed in germination so that we may be sure that we are not planting worthless seeds? What is the best method of storing seeds in order to maintain their full vitality? We believe our laboratory during its 14 years of existence has made progress in answering these questions for many kinds of seeds.

A few years ago Dr. Ohga discovered old Indian lotus (*Nelumbo nucifera*) seeds in a layer of peat about five feet below the surface of a naturally drained lake bed in Manchuria. By calculating from the erosion rate of the river, following up the geneology of a Chinese family that had farmed a portion of the drained lake bed for many generations, and by studying the annual rings of trees growing on the lake bed after its drainage, he came to the conclusion that these seeds must be several centuries old. In spite of their great age they produced 100 per cent of vigorous seedlings when the coats were broken and the seeds placed in water. The seed coats of these seeds are impervious to water, so the seeds were kept dry each in its own cell during all the years. Under this condition the

protoplasm of the embryos is stable enough to endure for centuries.

It is interesting to learn what portions of *Nelumbo* seed coats exclude water. Experiments have shown that the impervious layer consists of the outer one-fifth of the rather thick seed coats. All portions of the seed coat below this absorb water readily. The coat consists of about 20 layers of cells and of these only the outer layer of cells (epidermis) and the outer half of the second (palisade) layer of cells are impervious. At the middle of the palisade layer is a line clearly visible in sections under the microscope called the "light line." The "light line" is the inner limit of the impervious portion of the coat. If the outer portion of the coats are removed down to this line the seeds will swell when placed in water. In the old seeds the epidermal layer and the outer end of the palisade cells had been eaten away by fungi and bacteria nearly down to the "light line." No doubt the seeds with less resistant coats had already sprouted during the century of storage and the seedlings perished because of deep burial. Probably a few seeds had sprouted from year to year throughout the centuries.

This is not an isolated case of delayed and distributed germination of seeds in soil although it is an extreme case. Beal buried 21 kinds of weed seeds out-of-doors in moist sand in 1879. Tests of these seeds were made, first, at five-year periods and

tinue up to 160 years. The last test was for the 50-year period and showed the following five sorts of seeds still alive and giving the percentage of germination indicated after each: yellow dock, 52%; evening primrose, 38%; moth mullein, 62%; black mustard, 8%; and water pepper, 4%. The U. S. Department of Agriculture is running a very extensive set of experiments on seeds buried in soil. The last test reported was for the 20-year period. All kinds of cereal and most kinds of garden seeds had perished even after one year of burial, but many sorts of weed seeds showed high germination after 20 years in the soil. Some seeds of the following cultural plants were still dormant and alive after 20 years in the soil: timothy, Kentucky blue grass, several clovers, tobacco and celery.

Is there an advantage to wild plants in having the germination of a given crop of seeds distributed over years, or is this one of nature's extravagances? There is certainly a survival advantage so far as the plant is concerned. The soil is always well supplied with dormant seeds which are ready to produce plants whenever the soil is stirred up or otherwise cleared of competing vegetation. The effectiveness of the scheme is increased by the fact that stirring the soil not only removes competing vegetation, but it gives the seeds conditions that arouse them from their dormancy — more moisture, higher temperature, better oxygen supply, and in some cases, light.

If delayed and distributed germination is of advantage to the plant in nature, how does it serve the gardener or horticulturist? It annoys the grower markedly in two ways: 1. Once the garden soil is filled with weed seeds, years of cultivation are required to get rid of them even if no more are added by plants growing in the garden or brought in from the surroundings, and 2. When the grower wants to produce plants from seeds showing delayed or distributed germination, he meets the greatest difficulties. For many years the author and co-workers have been studying delayed germination of seeds to find out, first, the mechanics by which seeds are delayed in germination and to learn, second, how to

eliminate or greatly shorten this delay in such a way that practically all the seeds of a given crop will germinate simultaneously rather than be distributed over weeks, months, or years. Reasonably prompt and especially simultaneous germination of the whole crop are necessities for the practical grower.

As a result of these studies several classes of seeds have been defined as to the causes of their delay and methods worked out for shortening or completely overcoming the delay.

Hard coated seeds. We have mentioned above that the lotus seeds remained ungerminated in moist soil for years because the coats prevented absorption of water. Hard coated seeds are especially common in the legume or pulse family. The gardener learns this if he attempts to grow wistaria, Scotch broom, locust, Kentucky coffee-bean, or many other legume seeds. The farmers often have trouble with clover, alfalfa and other forage legumes. Several methods have been developed for overcoming the delaying effects of hard coats such as filing, hot water or sulphuric acid treatment and passing seeds through specially designed abrading machines. The abrading machines are especially made for handling large quantities of clover, alfalfa, locust and some other seeds. The seeds with greatest life span are those with hard coats. The coats protect the embryos against the action of moisture and oxygen of the air. Some such seeds have been found to maintain their vitality in herbaria and seed cupboards for a century and a half or more.

Coats restrict oxygen supply. The author showed that the upper seed of the cocklebur germinates later (generally a year later) than the lower seed because the thin seed coats of the former restrict the supply of oxygen to the embryo. A few other seeds are delayed in germination because the coats reduce the oxygen supply to the embryos. Lettuce, ragweed and other seeds become dormant if they are put into a germinator at 87° F. or above. This is due to the seed coats limiting the oxygen supply to the embryos.

Light sensitive seeds. Some seeds require light for germination and others are prevented from germination by light, while most seeds under proper conditions for germination are indifferent to light. Probably the reason the tobacco, celery and blue grass seeds remained dormant for 20 years in U. S. Department of Agriculture buried seed experiments was their need for light. Some seeds are extremely sensitive to light. Swollen tobacco seeds need little more exposure to light to induce germination than is given a photographic film. Swollen lettuce seeds are almost as sensitive. Blue grass seeds, on the other hand, require strong light for a long time to induce germination. *Lobelia inflata, L. cardinalis* and *L. siphilitica* seeds require light for germination, while some other species of *Lobelia* seed are indifferent to light. *Gentiana andrewsii* seeds are benefited by light, while *G. crinata* and *G. acaulis* will not germinate without low temperature stratification. These and other seeds requiring light are sown on the soil with little or no soil over them.

Low temperature stratification. Many seeds of the temperate zone need a period in a moist medium at a low temperature to after-ripen them for germination. In nature the winter supplies the low temperature and prepares the seeds for germination in the spring. The best stratification temperature varies for different kinds of seeds from 33° F. to 50° or 55° F. and the time, from a month to nearly a year. Among seeds requiring low temperature stratification are many rosaceous forms, dogwoods, and forest tree seeds, including some conifers, as well as seeds of many alpines, temperate zone wild flowers and water plants. Chemical and enzyme changes brought about in seeds by low temperature stratification have been worked out in some detail. These chemical and enzyme changes seem to be necessary to enable the embryos to break the coats and produce thrifty plants.

Many seeds that respond to low temperature stratification have partially dormant embryos. This is true of most rosaceous seeds studied, witch-hazel and many others. When these embryos are freed from all coats and placed in germinators, they show very sluggish growth in contrast to the vigorous growth of removed embryos from seeds that have been stratified. The tops of seedlings grown from the dormant embryos are striking dwarfs. If continuously grown at 60° F. or above they remain as dwarfs for six months to a year and a half, after which one or more buds start rapid normal growth. At any time one or two months of exposure of these dwarfs to 45° F. or lower temperatures will throw them into vigorous growth. In short a low temperature after-ripens the buds of dwarf seedlings as it does the embryos within the seeds.

Two-year seeds. Every nurseryman knows that many seeds when planted in the spring will not produce seedlings until the following spring and some of the seeds of a given planting may produce seedlings the second or third spring after planting.

There are at least two types of so-called two-year seeds. In the first type bacteria and fungi partially eat away the thick coats during the warm weather of summer so the embryos get an adequate supply of water and oxygen for after-ripening during the cold of winter. Among plants with such seeds are the common snowberry, silver bell tree, hawthorns with heavy woody coats, basswood, and most Cotoneasters. Such seeds can be forced to grow the first spring after maturing by removing the hard coats or partially eating them away with sulphuric acid followed by low temperature stratification to after-ripen the embryos.

In another type of two-year seeds the root grows during the first summer but the top will not start until the epicotyl or bud that forms it has been exposed to a low temperature for a while. Among plants that produce such seeds are peonies (especially tree peonies), colder temperate zone Viburnums, some lilies, Solomon's seal, and others. Umbrella pine seeds belong to this group, but differ from those just mentioned in that the two leaf-like cotyledons and the epicotyl are pushed above the ground by the lengthening hypo-cotyl. The seedlings

stage until exposure to cold after-ripens the epicotyl which then unfolds to form the little tree. Seeds of this type can be made to produce seedlings the spring after harvest by planting them in flats in a greenhouse soon after harvest and exposing the flats to low temperatures for a month or two in the spring after the root system has formed. Some of the Viburnums cannot now be handled in this way because the roots are so slow in starting. No doubt this slowness in root development can be overcome by further research. It is likely chemical or other treatments can be used to hurry up the growth of the roots.

Secondary dormancy. Many kinds of seeds when placed in soil of germinators furnishing very unfavorable conditions for germination become so dormant that they will not germinate when transferred to favorable conditions. This is true of lettuce seeds when put to germinate at temperatures of 90° F. or above, of some light-requiring seeds in dark germinators, of certain dark-requiring seeds in lighted germinators, and of some seeds in germinators at too low temperatures. Seeds in secondary dormancy can be aroused to germination by various special treatments, such as breaking the seed coats or in many cases by low temperature stratification. Poor stands of some

flower and vegetable crops are caused by unfavorable conditions throwing the seeds into secondary dormancy, and no doubt the long rest of some seeds in soil is due to secondary dormancy.

Other methods of hastening germination. Aside from 'the above classes of delayed germination some seeds show other peculiar requirements for germination. Many slow germinating seeds are brought into rather prompt germination by daily intermittent temperatures such as 60° F. for 18 hours of each day and 96° F. for six hours. Among seeds that respond to daily intermittent temperatures are blue grass and some other lawn grasses, celery, lettuce and many others. Some seeds require low temperatures for germination. This is especially true of alpines. Most of them will germinate at or near the freezing point. *Camassia Leichtlinii* Wats. will not germinate well much above 41° F., and *Lewisia rediviva* Pursh. shows little germination above 50°F. Even annual delphiniums show poor germination above 59° F.

A quick viability test for dormant seeds. Miss Flemion of this Institute has recently developed a method by which she can determine the viability of even the slowest germinating seeds within a week or two.
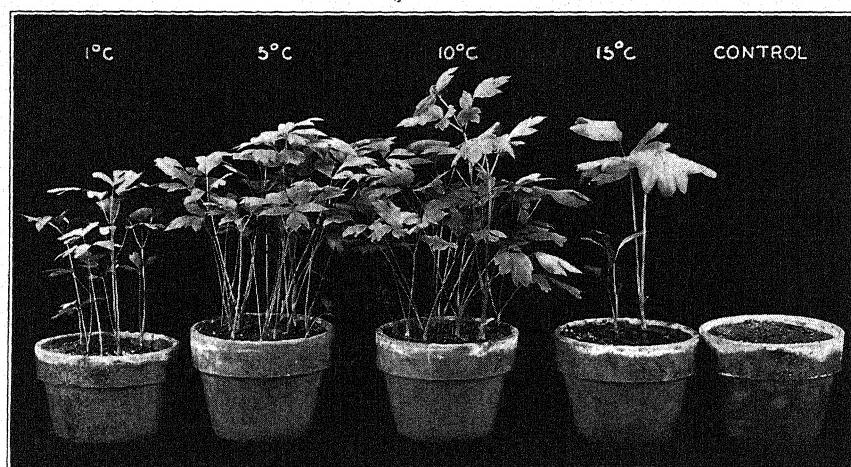
Low temperatures break epicotyl dormancy in tree peonies. Pots, containing seeds with roots already formed, were placed at various temperatures for two and one-half months then transferred to a greenhouse for shoot production. The control pot remained in the greenhouse for the entire period.

Annual Delphinium Seeds of Crop of 1926

Percentage Germination When Put in Storage, 72

Storage condition	Percentage germination after mos. in storage					
	11 mos.	22 mos.	46 mos.	69 mos.	111 mos.	123 mos.
Open room temp.	57	44	0	0	0	0
Sealed room temp.	75	80	50	15	0	0
Open 8° C.	50	41	31	5	0	0
Sealed 8° C.	70	45	66	80	76	71

The embryos are removed and placed on moist filter paper in petri dishes. The live embryos enlarge and in light become green, while the dead ones become brown and decay. This method should prove of great value to the seed industry, especially for slow germinating seeds.

Storage of seeds. Many seeds offer practical difficulty because of their short life span. Much attention has been given at the Institute to proper storage conditions for short-lived seeds. Some short-lived seeds need special storage conditions to prolong their vitality even for a year, but all seeds that can be thoroughly dried seem to be similar in their storage requirements. Proper drying followed by sealed storage in absence of oxygen and at a low temperature represents the optimum storage conditions for the latter group of seeds. The accompanying table shows how much sealed storage at low temperature will lengthen the life span of annual delphinium seeds. In open storage at room temperature these seeds had degenerated considerably in 11 months, whereas in sealed storage at 48° F. they showed no degeneration after more than 10 years of storage. The long-lived hard-coated legume seeds mentioned above are really in sealed storage. In them the impervious coats seal the individual seeds against exchange of oxygen and moisture with the outside atmosphere.

Our investigations indicate that for most garden and flower seeds that are to be kept only a year or two, storage in bags in a room with low but constant humidity is adequate without control of temperature or removal of oxygen. Even control of humidity of the air is an expensive process, but it can be done with profit by a large seed merchant who handles tons of seeds. The amateur gardener with small quantities of seeds to handle should dry the seeds thoroughly and store them in a tightly corked bottle in the refrigerator.

Boyce Thompson Institute delayed germination and seed storage project. This Institute has a project on seeds which employs five scientific workers continuously handling difficulties in germination and storage of seeds referred to them by gardeners, florists, conservationists, foresters and others. A score or more new seeds offering difficulties are added to the list for study each year. The investigators have developed largely the knowledge of four of the several classes and sub-classes of delayed germination mentioned above and are adding seeds to each class yearly with directions for producing reasonably prompt germination of each kind of seed. In these studies the attempt is made to develop methods that require no special equipment, but that can be applied by the practical grower with the equipment at hand. The best methods for the storage of many sorts of seeds in order to lengthen their life span have been studied and more seeds are being added continually to this list.